

Identification and validation of a vitamin D-related prognostic signature in colorectal cancer

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Abstract

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1. Introduction

Colorectal cancer (CRC) is the third most common malignant tumor worldwide and is the second one in cancer-related deaths [1]. In spite of improvements in the management and treatments of patients with CRC in the last two decades, no satisfactory therapy exists when the surgery is not curative. The poor prognosis and the increasing incidence of CRC have provided strong motivation to construct a predictive model in CRC patients, which will benefit personalized treatment in clinical management [2]. There are lot of epidemiological and preclinical studies that indicate a beneficial effect of vitamin D on CRC incidence and mortality [3] [4]. Vitamin D is a fat soluble vitamin and many genes are related to its metabolism and action [5]. It can be obtained from diet or the endogenous synthesis in the epidermis under sunlight exposure [6]. It

has been demonstrated that vitamin D benefits clinical outcome and improves the long-term survival of CRC patients [7]. Moreover, circulating vitamin D may be a CRC biomarker and its deficiency is related to the high incidence of CRC [8]. A better survival outcome in CRC is associated with higher prediagnostic or postdiagnostic serum 25-hydroxyvitamin D concentrations [9]. The most active vitamin D metabolite (1 α ,25-dihydroxyvitamin D3) inhibits the proliferation and promotes the differentiation of cultured colon carcinoma cells by mechanisms that include cell cycle arrest at G0/G1 phase, blockade of the Wnt/ β -catenin pathway and induction of Ecadherin and other epithelial proteins [3] [10] [11]. Lots of genes related to vitamin D metabolism and action play an essential role in tumors. For example, CYP24A1 an important vitamin D-related gene, is up-regulated in CRC patient and nominated as a promising biomarker [12]. Vitamin D and its related genes are correlated with the homeostasis of the intestinal epithelium and regulate immune cells [13].

The objective of this project was to find a way to make prognosis and stratify patients suffering from colorectal cancer by means of their transcriptomic profiles. In particular we focused on the gene signature of vitamin D as a prognostic marker, by leveraging the always increasing gene expression data publicly available. The final goal was to identify colorectal cancer survival markers related with vitamin D effects, as well as any pathways involved.

2. Material and methods

2.1 Data preprocessing

To achieve a better statistical significance, a high number of samples was originally collected from different available public datasets on gene expression in CRC. These were downloaded from the databases Gene Expression Omnibus (GEO) [cite] and The Cancer Genome Atlas (TCGA) [cite]. In total, 21 datasets from GEO and 1 from TCGA were obtained, and their metadata manually curated and standardized (table 1). It is worth noting that only the dataset GSE157982 had the gene signature of Vitamin D in CRC, necessary for its further

analysis.

2.1.1 Sample splitting and filtering

In order to filter the samples having all the data necessary for the downstream analyses, they were split in 6 sets according to their available clinical data. Metadata to be considered was, for instance, survival time and tumor stage. After this operation, 2676 samples were retained out of the starting 3072 (87.11%), divided as in table 2.

Set usage	n° of samples
COX fitting	388
KM curve	157
Vitamine D low	49
Vitamine D high	49
Stage low	1120
Stage high	908

Table 2. Split samples

2.1.2 Dataset normalization

Having decided to use different dataset for this analysis, a normalization step has been introduced to remove batch effects. To do so we performed on the raw micro-array data different type on normalization. Firstly we tried with Robust Multiarray Analysis *RMA* and then with Frozen Robust Multiarray analysis *fRMA*. The logarithmic value of all the gene expression levels has been computed and used downstream the analysis. We divided the normalized data in two sets to be used downstream. The first was composed of datasets GSE17536 and GSE17637 and will be used to fit the Cox proportional-hazards model. The second was composed of datasets GSE31595, GSE33113, GSE38832, GSE39084 and GSE39582. This second set has been normalized in two ways: we first normalized all the samples together to compute a single data matrix used downstream to plot the Kaplan-Meier curves and then we implemented a bootstrap procedure. In this procedure we sampled from each dataset 600 individuals 10 times so that in each matrix we obtained balanced data with respect to the cancer stage.

2.2 Differentially expressed genes

After having removed the batch effect and having obtained all the necessary datasets we used them to find two sets of differentially expressed genes or *DEGs*. The first set contains DEGs found in different stages of cancer and the second the vitamin-D gene signature.

2.2.1 DEG between stage of cancer

To find DEGs between the stage of cancer we used 10 data matrices of 600 samples. This data matrices have been computed so that the samples were balanced between high and low stage. We identify stage low of cancer as stages 0, 1 and 2 and stage high as stages 3 and 4. For each of the 10 subsets we used LIMMA to find a ranking of the DEGs. The resulting gene lists have been sorted in increasing order by p-value. We aggregated the resulting list using Borda count, so to obtain a

ranking for each of the *temp* genes. This ranking will be used to compare prediction power of the vitamin-D signature with respect to the top DEGs between stages.

2.2.2 Vitamin D gene signature

In order to obtain the list of differentially expressed genes for the vitamin D signature, the state-of-the-art R package “Deseq2” was used on the vitamin D dataset, which contains counts of RNA-seq expression data from rectal biopsies of CRC patients pre-and post- vitamin D supplementation. Transcript IDs were converted to Gene Symbols through “BioMart”. For some of the transcripts, a Gene Symbol was not found. The statistically relevant genes were then selected using the adjusted p-value automatically computed by the Deseq2 package. DEGs were then manually expanded using the protein-protein interaction network database STRING. Enrichment analysis was performed on the original list using the web interface EnrichR.

2.3 Survival analysis

The survival analysis has been used to determine if both sets of DEGs we found upstream are responsible for a change in the probability of survival. This analysis has been performed in two steps. The first uses a cox proportional-hazards model and the second involves building the Kaplan-Meier curves for the significative gene found by cox on a different dataset.

2.3.1 Cox proportional-hazards model

Cox-proportional-hazards model is used to determine for each of the *temp* gene if they are significant in changing the overall survival and the corresponding hazard ratio *HR*. The hazard ratio determines how a gene is associated with the length of survival. For each gene the optimal cutpoint for the level of expression will be computed using the *cutp* function. This model has been fitted using dataset TEMP and were considered significative genes with $pvalue \leq 0.05$.

2.3.2 Kaplan-Meier curves

For each one of the significative genes found by cox a Kaplan-Meier curve was computed using data from dataset TEMP. One of the two curves of the plot plotted samples with expression level less than the cutpoint, while the other expression level greater than it. The gene were still considered downstream if the log-rank p-value was still ≤ 0.05 . The list of genes that survived this process was intersected with the enriched vitamin D signature and the DEG ranking.

3. Results

3.1 Data preprocessing

3.1.1 Sample splitting and filtering

3.1.2 Dataset normalization

3.2 Differentially expressed genes

3.2.1 DEG between stage of cancer

3.2.2 Vitamin D gene signature

A serum level of vitamin D was chosen as a threshold for stratifying the two groups of patients (high vs low vitamin

Dataset name	Sample description	Number of samples
GSE157982	baseline and vit. D-treated CRC rectal samples	98
GSE38832	tumor colorectal samples	122
GSE14333	tumor colorectal samples	290
GSE17536	tumor colorectal samples	177
GSE31595	tumor colorectal samples	37
GSE33113	tumor colorectal samples	96
GSE38832	tumor colorectal samples	122
GSE39084	tumor colorectal samples	70
GSE39582	tumor colorectal samples	585
GSE103479	tumor colorectal samples	156
GSE17537	tumor colorectal samples	55

Table 1. Starting datasets

D level). This is because for a small subset of patients in the post-supplementation group, the serum levels were relatively low, whereas for another subset of patients in the pre-supplementation group, levels were already high. The chosen cutoff eventually led to better results than the classification based on the available label. Principal component analysis on the full expression data matrix did not yield segregated clustering of the two groups. However, in order to perform enrichment analysis, the top 100 differentially expressed transcripts for which a Gene Symbol could be found were given as input to EnrichR.

3.2.3 Pathway enrichment

3.3 Survival analysis

3.3.1 Cox proportional-hazards model

Cox proportional-hazards model identified from the initial *temp* genes 943 genes.

3.3.2 Kaplan-Meier curves

Of the 943 genes found significant by the cox proportional-hazards model 291 has been found significant on the dataset used to build the Kaplan-Meier curves. Of these 291 4 are found in the enriched vitamin D signature (left side of figure 1). To compare how this vitamin D related gene have an impact on the probability of survival we compared them with the top 4 DEGs identified between stages of cancer and found significant in this step (right side of figures 1).

For each of these gene the respective *HR* can be found in table 3

Vitamin D gene	HR	Stage DEG	HR
RPS27A	$1.4 \cdot 10^8$	SLC22A8	$3.3 \cdot 10^6$
TMCO3	$7.9 \cdot 10^5$	CDK17	$5.2 \cdot 10^3$
CHUK	88	DHRS12	$2.9 \cdot 10^3$
KLHL20	$1.6 \cdot 10^{-6}$	AMPD3	$2.6 \cdot 10^{-7}$

Table 3. HR for significant genes

4. Discussion

After having compiled the Kaplan-Meier curves the four genes derived from the consensus stage-based DEGs and the four gene found from the expanded vitamin D gene signature found statistically significant, we compiled a literature-based characterization for each of those (tables 4 and 5).

Gene	Relevant function
RPS27A	One of the genes encoding for ubiquitin.
	Misregulated in various cancers, including colorectal [n].
	Its upregulation in CRC may promote cancer cell proliferation and inhibition of apoptosis [o].
	Probable Na(+) / H(+) antiporter. Linked to unfavorable outcomes in cancer [p].
TMCO3	A patent exists for prognosis and treatment methods of CRC based on this gene [q].
CHUK	Ser/Thr protein kinase involved in the (indirect) activation of NF-kB [f].
	Regulates cyclin D1. [g]
	Decreased activity is linked to cancer [h].
KLHL20	[i] Mediates ubiquitination of DAPK1 thereby downregulating interferon-mediated apoptosis. [l]
	Mediates ubiquitination of PML thereby promoting resistance to hypoxia and cancer progression through HIF1a signalling. [m].

Table 4. Statistically significant genes in the expanded vitamin-D gene signature

References

Gene	Relevant function
SLC22A8	Integral membrane protein involved in the sodium-dependent excretion of potentially toxic organic anions. Expression specific to kidney and brain [e].
CDK17	Cyclin-dependent protein serine/threonine kinase. Involved in the regulation of transcription involved in G1/S transition of mitotic cell cycle (source: GO biological process). Expressed in many tissues (no specificity) [b]. It is the target of 4 CDK inhibitors.
DHRS12	Oxidoreductase. Linked to poor prognosis in ovarian cancer [c] and suppression of proliferation and metastasis in osteosarcoma [d].
AMPD3	AMP deaminase in erythrocytes. In mice, mutations on this gene reduce levels of naive CD4+ and naive CD8+ cells in peripheral blood but not in lymphoid tissue. This is most likely due to a signalling mechanism triggered by the mutated phenotype of the red blood cells. [a].

Table 5. 4 top genes in stage DEGs

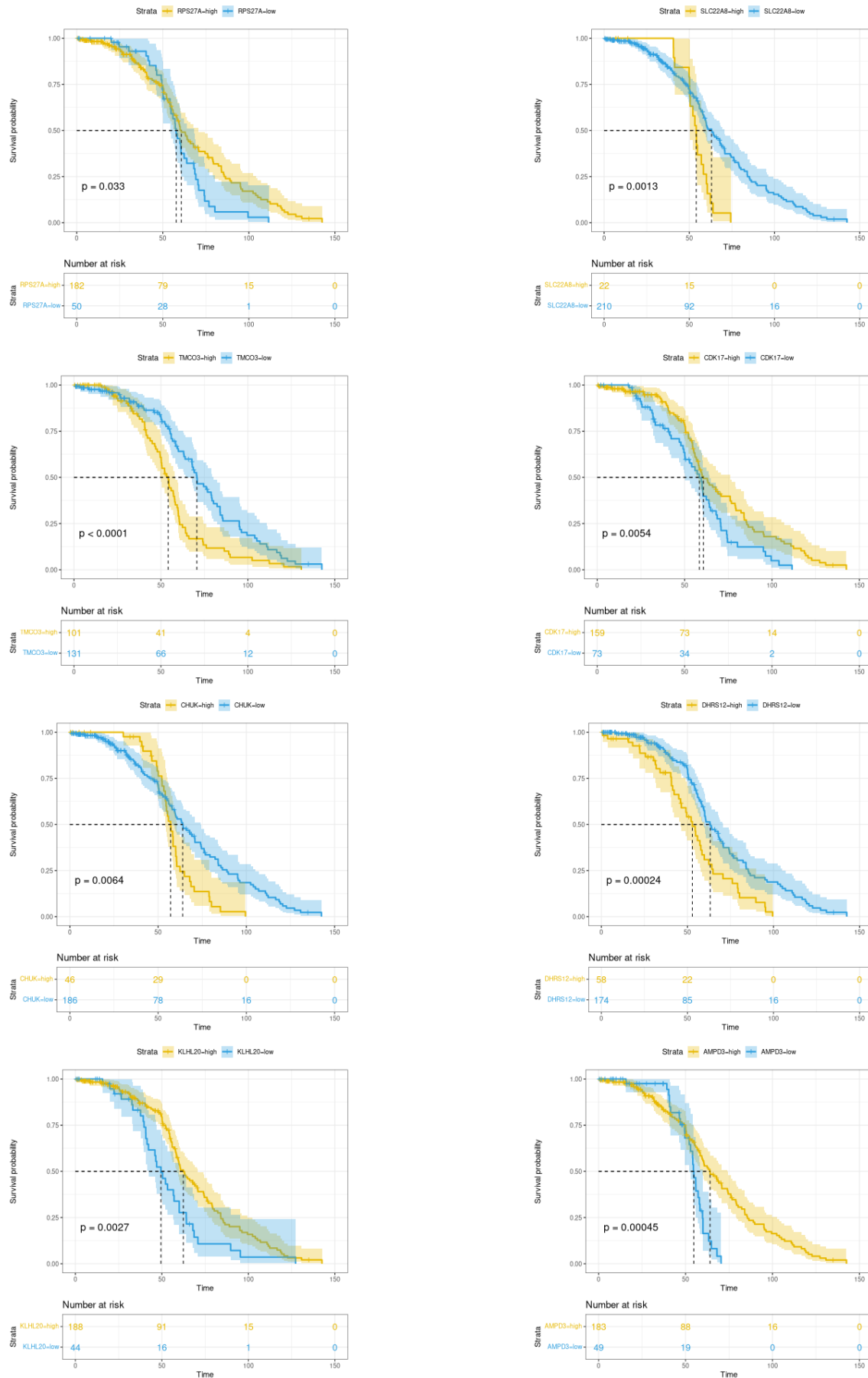


Figure 1. Survival curves, on the left vitamin D related genes, on the right the top stage DEGs