

The Database dbEST Correctly Predicts Gene Expression in Colon Cancer Patients

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Abstract: This study aims to test the predictive power of gene expression data derived from NIH's database dbEST, which collects gene expression results from a large number and variety of DNA array experiments. The motivation of this study is to make comparable experimental studies, which are usually performed only for one or a few tissues or organs, with a wide variety of other tissues. Confirmation of a good predictive power of dbEST would put a number of interesting and partially surprising recent findings, solely based on data mining, on a more solid basis than available so far.

The expression of nine genes (eIF4E, DDX6, HAT1, USP28, HSP90 β , PKM2, PLK1, COX2 and OPN) plus two calibration genes in paired normal and cancer colon tissues of eight individual patients was investigated by quantitative RT-PCR and compared with the predictions made by the data - base. GUS and β -actin reveal only little variation among different patients, making them good internal calibration standards. In normal colon tissue, data mining correctly predicts the expression of all nine genes, which covers two orders of magnitude. In cancer, dbEST is somewhat less precise, but still valuable for the comparison with clinical results.

†This paper is dedicated to the memory of Beatrice Louise Pool Zobel who passed away on May 13th, 2008. Beatrice has initiated the experimental part of this work.

INTRODUCTION

The first steps in the development of new drugs can be made significantly more efficient by data mining. One hallmark of many cancers is the degree of expression of given genes, which may be studied with DNA microarrays. Millions of such data are available in the database dbEST, provided by the NIH. Employing this database is particularly useful, when not only one or a few tissues are of interest, but an overview over a wide range of tissues is the aim. (See also Discussion in the present work). A drawback so far was that the findings were solely based on data – bases. Therefore drug developers may hesitate to accept that the findings are meaningful for true patient data. The aim of the present communication is to compare data predicted by dbEST with real human patient data and to check how good such predictions can be. Since it is generally difficult to get paired normal and cancer tissue from human individuals, a cancer type had to be chosen where such material can be obtained comparably well. Such a tissue is colon, since in colon surgery usually normal material is excised together with the tumour.

MATERIALS AND METHODS

The Investigated Genes

In the following a short description of the genes selected for the present study is given:

Osteopontin (OPN) is a glyco - phospho- protein involved in processes such as cell migration and proliferation, cell survival promotion and apoptosis protection [1-3]. Within the cell OPN forms a functional tandem with PLK1, and induces COX2 (see below). Moreover, OPN promotes a number of cancers with a prominent role in colon cancer malignancy and metastasis [4-6].

Cyclooxygenase 2 (COX2), unlike COX 1, is the inducible form of the enzyme, involved in prostaglandin synthesis and thus promotes tumour cell proliferation, survival and angiogenesis [7-9]. Elevated COX2 expression is found in 80 % to almost 100 % of the colon cancers [10-13].

DEAD box 6 (DDX6), also named as *RCK* or *p54* modulates the mRNA secondary and tertiary structure and has thus been postulated to facilitate translation and thus indirectly proliferation and malignant transformation [14, 15].

Eukaryotic initiation factor 4E (eIF4E) is a translation factor the activity of which regulates the level of translation initiation. Elevated eIF4E expression may contribute to increase in both, general protein synthesis and synthesis of proteins promoting the cell growth and the angiogenesis which consequently leads to neoplastic transformation [16, 17].

Histone acetyl transferase 1 (HAT1) is involved in histone modification and thus in maintenance of the chromatin structure and the viability of the cells. Disturbance in the covalent histone modification may be implicated in the development

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of neoplasia [18, 19]. In that function, HAT1 is thought to be relevant in cancerogenesis, but also in ageing.

The *ubiquitin specific protease (USP28)* is required for the stabilization of the MYC proto-oncogene. Reduction of its expression can strongly inhibit the growth of cancer cells [20].

Polo-like kinase 1 (PLK1) phosphorylates/activates cell cycle proteins thus driving cells into mitosis [21, 22]. It is also involved in centrosome maturation and assembly, spindle function, mitotic exit and cytokinesis and therefore its inactivation may contribute to mitotic arrest, induction of pro-apoptotic pathway and suppression of tumour growth in response to stress [23]. PLK1 over-expression may lead to elevated proliferation and cellular transformation [24].

Pyruvate kinase, muscle (PKM2) is glycolytic enzyme, predominantly expressed in cells with increased nucleic acid synthesis, as for example proliferating cells like those from the fetal tissue, adult stem cells and tumour cells [25, 26]. It was shown that the level of tumor PKM2 correlates with the ability of tumour cells to form metastases [27, 28].

Heat Shock protein 90 beta (HSP90 β) acts as a “molecular chaperone“ under normal physiological conditions thus is critical to maintaining the normal protein folding environment and therefore is able to modulate the protein activity. It affects the arrangement of the protein complexes, the protein translocation and degradation by the proteasome pathway and thus affects the apoptosis. Therefore, its altered usage reflects the ability of the malignant cells to maintain homeostasis in the host environment [29, 30].

Tissue Samples from Patients

All patients have given their informed consent and the study was institutionally approved by the ethics committee of the Friedrich – Schiller University of Jena. Altogether, tissue samples from eight patients were included in this study. The donors of the colon tissue were admitted to the University Hospital of Jena, Germany to undergo surgery for removal of colon tumours or colon polyps. The normal colon samples from each patient with colon cancer were taken in a distance from the tumour site. Confirmation of the tumour stage of the patients was provided by pathological examination after the surgery (Table 1).

The tissue samples were stored in Hank's balanced salt solution (HBSS – 0.8 g/L NaCl, 0.4 g/L KCl, 0.06 g/L Na₂HPO₄ x 2 H₂O, 0.06 g/L K₂HPO₄, 1 g/L glucose, 0.35 g/L NaHCO₃ and 4.8 g/L HEPES, pH 7.2) and transported to the laboratory on ice within 1 h after the surgery. The human colon epithelium was separated from the tissue by perfusion-supported mechanical disaggregation [31]. The epithelial stripes were immediately submerged in RNeasy (Qiagen, Hilden, Germany), stored at - 80°C until further use for RNA extraction. The median age of the patients was 71 years (range 57 years -84 years). The group consisted of 6 males and 2 females

Total RNA Extraction

Total RNA was isolated from the colon tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) where ge-

Table 1. Clinicopathologic Characteristics of the Eight Patients Whose Paired Types of Tissues were Examined for Expression of Nine Genes Implicated in Various Cellular Processes

Patient and tumor characteristics	Number of cases
Analyzed donors	8
Healthy	8
Disease	8
Sex	
Male	6
Female	2
Age (years)	
Mean age 71.44 ± 8.02	
Type of analyzed tissue	
Normal	8
Tumor	8
Tumor grade	
UICC I	1
UICC II	4
UICC III	3
UICC IV	none

nomic DNA contaminations are effectively removed by using gDNA -Eliminator spin column. Two different ways for disruption of the colon stripes placed in RNeasy solution were applied. Either the stripes were grounded in liquid nitrogen and homogenized by using pellet pestle motor. Or Polytron PT 2100 homogenizer (Kinematica AG, Littau-Luzern, Switzerland) was utilized. An appropriate volume of RLT Plus buffer was added to the tissue powder or the colon stripes for further lysis and/or homogenization. Next steps of the RNA isolation were performed according to the manufacturer's instructions. The RNA yield and the ratio of absorbance at 260 nm to 280 nm (A₂₆₀/A₂₈₀ ratio) was measured with the NanoDrop[®]ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). Prior to *in vitro* reverse transcription steps the integrity of the isolated total RNA was checked by agarose-formaldehyde denaturing gel electrophoresis or for more rapid and precise characterization of the isolated total RNA the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used. The RNA integrity number (RIN) of the analyzed samples, determined by this technique was between 6.4 and 9.6 (The classification of the eukaryotic total RNA is based on a numbering system from 1 to 10 as 1 corresponds to most degraded RNA and 10 to the most intact RNA).

cDNA Synthesis and Quantitative Real Time PCR

First strand cDNA was synthesized from 3 μ g total RNA using oligo (dT) 12-18 primers (Invitrogen GmbH,

Karlsruhe, Germany) and SuperScript II Reverse Transcriptase (Invitrogen GmbH, Karlsruhe, Germany) according to the provider. After synthesis, cDNA was diluted 10 times with RNase-free water and stored at -20°C till further processing. Quantification of the relative expression of nine target genes (PLK1, PKM2, OPN, COX2, eIF4E, DDX6, HSP90 β , HAT1 and USP28) were investigated. For each reaction 2 μL of cDNA was mixed with PCR master mix iQTM SYBR[®] Green Supermix (2xSYBR Green I, iTaq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, 10 mM MgCl₂, 20 nM fluorescein, and stabilizers, Bio-Rad), and the gene-specific primers in a final volume of 25 μL . The quantitative PCR assay was performed in duplicate or triplicate. The expression of two endogenous references, i.e., β -actin and β glucuronidase (GUS) served as an internal control for further careful normalization and for correcting results of different amounts of input RNA.

Primer Design

All primers were designed by using the freely available Primer3 software, version 0.4.0, (<http://frodo.wi.mit.edu/>). Notably, the primers were designed to amplify across an intron / exon boundary or in two different exons, separated by very big intron, thereby preventing amplification of residual genomic DNA. The amplicon size is between 94 bp and 202 bp.

PCR Conditions

The enzyme was activated after an initial two- minutes denaturation step at 95°C and 40 cycles of 30 sec at 94°C , 30 sec at 60°C and 30 sec at 72°C . Amplification was performed in an iCycler thermal cycling instrument (iCycler iQ[®] Real-Time PCR Detection System; Biorad GmbH München, Germany) in 96-well microtiter plates. For each experimental run, a control reaction that contains all essential components of the amplification reaction except template was included. This enables detection of contaminations and presence of primer-dimer formation.

PCR Reaction Efficiency

The slope of the standard curve provides an indication of the efficiency of the real-time PCR. The standard curve was generated using a dilution series of five different concentrations of cDNA, measured in triplicate. The standards were giving a slope between - 3.1 and - 3.4, which was acceptable for accurate quantification.

The fluorescent signal from each PCR reaction was collected as the peak-normalized values plotted versus the cycles numbers. Reactions were characterized by comparing Ct values. The Ct value is a unit - less value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above the baseline, when it is always located within the linear phase of amplification. The higher the initial copy number of cDNA for certain gene target is the lower Ct value this gene has. The qRT-PCR data were analyzed using comparative Ct method. The average Ct value for the reference gene minus the average Ct value of the gene of interest (ΔCt) represented as a log with a base two.

Data Mining

Expression data for these genes as well as the classification of tissues have been obtained via the "Virtual Northern" function of NIH's database dbEST [32-34]. Recent short descriptions are available in references[35-37]. This database searches in literature for data from DNA or oligonucleotide chip experiments, normalizes them and lists the results gene by gene for a set of 51 tissues (plus 3 pooled values). The data can be obtained for each gene via <http://cgap.nci.nih.gov/Genes/GeneFinder>. Presently, the database contains information on some 4 million gene or EST expression data.

RESULTS

In this study, an innovative combination of genes, never used before in the cancer diagnosis was assayed by quantitative RT-PCR (qRT-PCR) for their expressions in paired normal and tumor colon tissue. The selection of the entire set of genes was performed based on their altered expression during the development of various cancer types, including colon cancer. The proteins encoded by these genes are involved in a broad spectrum of cellular processes as translation initiation, cellular proliferation, mitosis, apoptosis etc. A detailed description of the 9 genes investigated in this work is available in the section *Material and Methods*. Moreover, the derived PCR data was compared with the data provided by dbEST database. Besides that, it is essentially impossible to obtain in different types of experiments, the absolute expression value for a given gene. Therefore, in order to make results from different patients and those from mining in databases comparable, a sort of normalisation is required. This, in turn postulates suitable normalization genes, which have constant expression levels in a very different conditions. For this purpose, the housekeeping genes are good candidates. In theory these genes are responsible for a basic cell survival and thus should be constitutively expressed in all cell types, without being affected by any human diseases or experimental conditions. In our investigation, as an endogenous standard β -actin was employed together with β glucuronidase (GUS). Indeed, Fig. (1) shows that the broadly used β -actin as well as GUS meet the requirements for normalisation genes. In eight different human individuals, they both need relatively the same number of PCR amplification cycles to become detectable. The fact that for the eight investigated individuals these values are the same in normal and cancer tissue is remarkable, but not mandatory. Even more, if in a specific individual, there are minor variations, they are the same for β -actin and GUS, so that the difference remains constant.

In addition, for validation of their similar expression patterns, a metric analysis such as a Pearson correlation test is applied. The analysis revealed significant co-expression of β -actin and GUS in normal ($p = 0.009$) and in tumor colon tissue ($p = 0.038$), respectively. A value of $p < 0.05$ is considered significant. This indicated that β -actin and GUS are equally suitable to be used as normalization genes.

In order to simplify the analysis, we selected β -actin for further calibration of the data. The relative expression derived by qRT-PCR was evaluated by using comparative Ct method (see the description in *Material and Methods*). The normalization of the expression data obtained from the

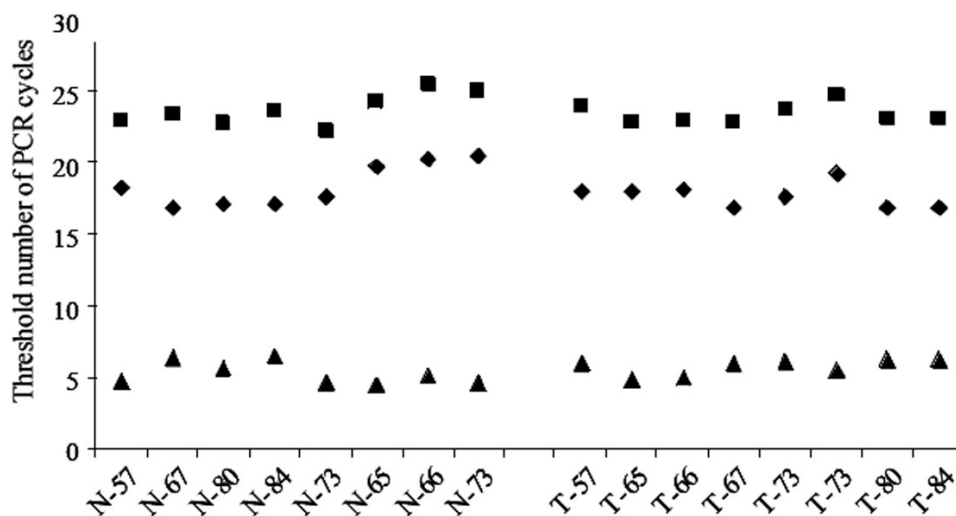


Fig. (1). Threshold number of PCR cycles required for finding first a fluorescence signal significantly above background of GUS (■), β -actin (◆) and difference thereof (▲) for the eight patients under investigation. The left part of the graph represents the expression of GUS and β -actin in normal tissue (N) and the right part shows the expression patterns in tumor tissue (T). Note that a change in Ct value by one cycle corresponds to a factor of two in expression level.

dbEST database was achieved by dividing the value of the gene of interest by the value of the selected reference gene. Once the matter of normalization is clarified, the data for other genes can be taken without further precautions, except that they have to be obtained with the identical procedure as the β -actin values. This does now allow direct comparison of patient data, generated by qRT-PCR with the database values. Generally, one can expect that normalized expression values for different genes are vastly diverse among the patients. This is shown in Fig. (2), where the expression data of nine investigated genes produced by qRT-PCR across the eight different patients was presented in combination with the data from the dbEST database. Here, the individual variability in the expression of the analyzed genes is depicted with a rhomb symbol (◆). For better visualization, the rhombs are connected. Furthermore, the horizontal lines represent the values derived from the dbEST database and are the average over typically hundreds of patients each. On the right (Fig. 2A and B) the results for normal colon tissue are given, on the left (Fig. 2C and D) those for cancer. The upper row (Fig. 2A and C) gives a representation of all nine investigated genes, where those genes with low expression level appear on the zero line. The lower row (Fig. 2B and D) gives the same data with another scale of the y-axis. Here the genes with low expression levels are better represented, while those with the highest expressions are out of scale in this representation.

Note that in total the expression levels vary by almost two orders of magnitude. In view of that, the predictive power of dbEST for normal tissue is excellent. For almost all genes the horizontal line for the prediction either intersects the line for the individual data points, or it comes close to them within a factor of two, which can, in terms of the general accuracy of DNA microarray data, be regarded as correct.

Only for HAT1 expression there is an apparent discrepancy. This is however, caused by the fact that in the dbEST

database no data for normal tissue are available and therefore in Fig. (2), its expression appeared on the zero line. As soon as data becomes available, the prediction value may reflect the experimental data better.

The predictive power of dbEST for cancer tissues is somewhat lower. One explanation, which could explain the disagreements, is that the database averages data over all subclasses of colon cancer while the qRT-PCR data were generated from cohort with a narrower spectrum of colon cancer stages. A summarized overview of the available clinico-pathological characteristics of the analyzed patients can be seen in Table 1 in *Material and Methods*. In spite of that, except for PLK1 and PKM2 genes the agreement is good and even the poorer results for the latter is still useful (see discussion)

DISCUSSION

The results of the present study open the path for the use of dbEST data in clinical studies. Though particularly only shown for the case of paired normal and cancer colon tissues, it is reasonable to assume that prediction of gene expression by data mining for other tissues may be also useful. This will allow researchers, with minimum effort, to check for example, whether a gene behaves as cancer gene only in the experimentally investigated cell line or tissue or behaves similar in a wide variety of tissues and organs. For example, our earlier findings related to the Warburg effect, at that time based solely on data mining, that some cancers exploit glycolysis considerably more than others [35], is now on significantly safer ground. One of the genes of glycolysis, PKM2, is even part of the present experimental study. In spite of the fact that, in colon cancer, it is the gene with the poorest agreement between prediction and experiment, it still correctly states clearly up regulation in cancer, in line with the result from our eight patients. Two other statements of multi tissue analysis we had made earlier solely by data mining, do now also come on a safer ground. In one study we

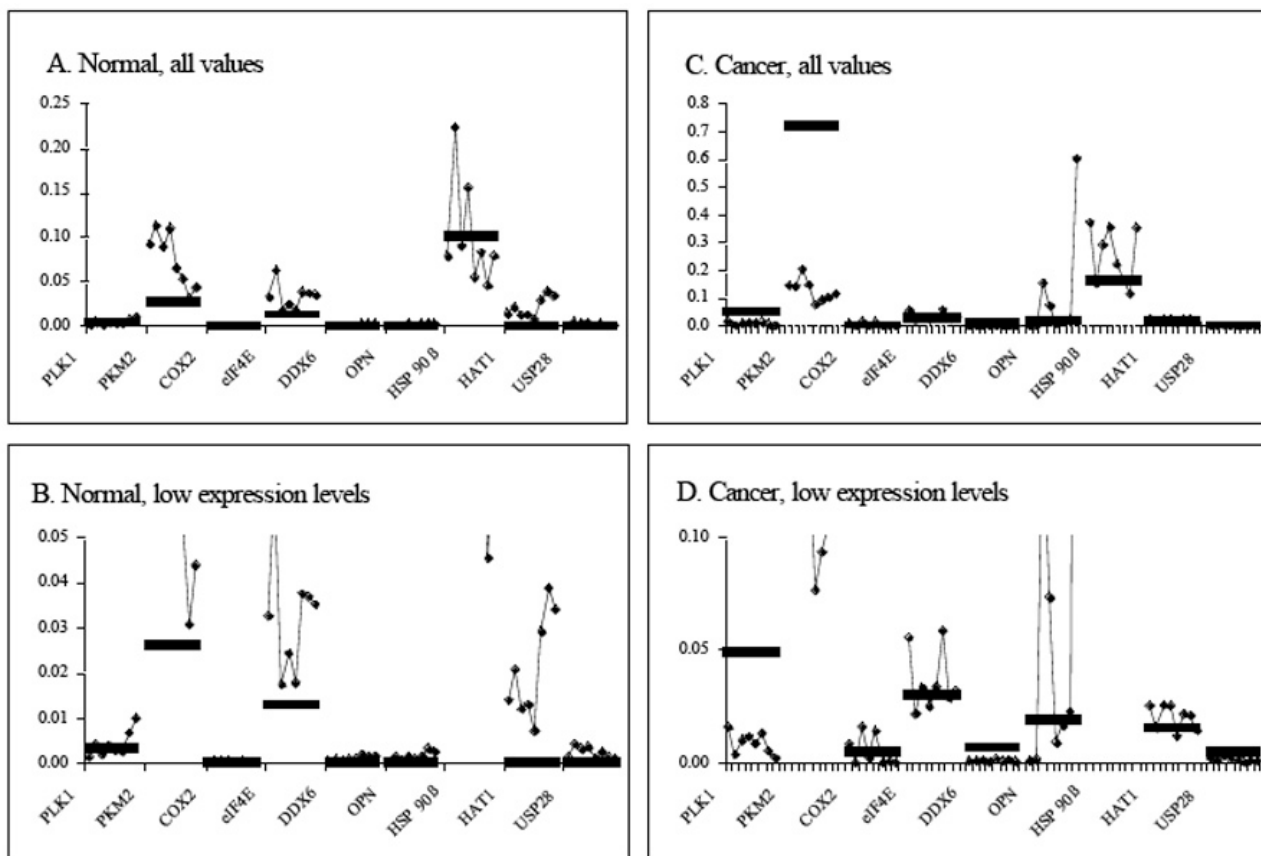


Fig. (2). Comparison of the generated by qRT-PCR patient data with the data from dbEST prediction. The linked to each other rhombs (◆) corresponds to the individual variation in the expression data for defined gene across eight patients, ordered by their age. Straight horizontal lines are predictions by dbEST; **A)** normal tissue, all genes; **B)** normal tissue, different scale on y axis in order to show genes with low expression; **C)** cancer, all genes; **D)** cancer, genes with low expression scale.

had found that only approx. 50 of the 20 000 known human genes are generally over- expressed in a majority of 24 different tissues [36]. In another study we had shown that the expression levels of genes for 62 binding partners of the tumour suppressor gene p53 add up to surprisingly similar values in 24 different tissues and that these sums are more uniform in cancer than in the corresponding normal tissues [37].

An application of the present results for future studies would be to classify the gene expression in different individuals, where there is a strong inter-individual variation, as for example for the heat shock protein HSP90 β in Fig. (2). Among the individual cancer tissues, some are closely in line with the prediction, others deviate significantly. This is not surprising when one considers the fact that the dbEST averages over hundreds of patients. Therefore, those of our patients where there is an agreement are "typical ones", while those with a high deviation are outliers, for whom one might even suggest a modified treatment of their cancer.

In conclusion, the present study has shown that dbEST indeed gives good predictions of gene expression for comparison with clinical data. Thus, the wealth of gene expression data buried in dbEST is more reliable than one might have expected so far.

ACKNOWLEDGEMENT

This work was supported by the Thüringer Ministry of Technology and Labour, grant B 309-05001 and by the Deutsche Forschungsgemeinschaft, grant Po 284/8-3 and 9. B. A thanks Dr.Toby Gibson for enabling her to perform the data mining work at the EMBL Heidelberg. We are grateful to Julia Sauer who gave us a good start into this subject.

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