In silico screening for tumour-specific expressed sequences in human genome

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Abstract A computer-based differential display tool named HsAnalyst has been developed and successfully used for the comparison of expression patterns in a set of tumours versus a set of normal tissues. A list of EST clusters highly represented in tumours and rarely observed in normal tissues has been developed as a resulting output file of the program. These differentially expressed EST clusters (genes) can be useful for developing new tumour markers and prognostic indicators for a wide set of human malignancies. Tumour-specific protein-coding genes may be considered a manifestation of tumour-specific gene expression. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Computer-based differential display; Cancer; Gene expression

1. Introduction

Comparing patterns of gene expression in different cell lines and tissues has important implications for various biological problems. The search for human genes specifically expressed in different tumours is one of the major challenges for modern tumour biology. A number of experimental methods are designed for tumour-specific gene search. Most of them are based on time-consuming and expensive experimental protocols (numerous modifications of the differential display approach, cDNA microarrays, serial analysis of gene expression) [1,2].

The total number of ESTs in publicly available databases ($>2\times10^6$) exceeds by approximately two orders of magnitude the total number of different transcripts that can be deduced from the number of human genes ($2.5–4\times10^4$) [3,4]. This provides a strong basis for the development of computer-based procedures for the subtraction of different EST pools instead of traditional experimental approaches used to compare expression profiles. An attractive method of in silico search for tumour-specific genes would be a 'computer-based differential display' (CDD), an analogue of experimental differential displays. The principle of CDD is to compare expression patterns in a particular tissue versus any other tissue source. This comparison is based on sequence databases publicly available in the World Wide Web. As an example of successful implementation of CDD the search for prostate-specific genes performed by Vasmatzis et al. [12] can be considered. A similar approach was also used for the search of differentially or ectopically expressed genes in particular tumour types in comparison to the corresponding normal tissue [13]. We tried to implement the CDD approach in the search for human tumour-specific genes. This gene-hunting procedure was inspired by the hypothesis that tumours might provide conditions for the expression of some genes that were not expressed in any normal tissue [5]. An experimental approach using combined preparations of mRNAs from several tissues in saturation and subtractive hybridisation experiments [6] led to the idea of subtracting all available tumour libraries against all available normal libraries instead of pairwise comparisons of each tumour and corresponding normal tissue.

Software instruments for CDD are available on NCBI WWW sites such as UNIGENE and Cancer Genome Anatomy Project (CGAP). These tools have built-in limitations that allow a researcher to operate only with pre-selected libraries. To overcome these limitations, we used the complete set of human ESTs compiled in dbEST database as archived flat-text files and UNIGENE information concerning EST clusterisation by perfect homology. By the time our search was initiated (January 2001) the database included about 2.2×10^6 individual human cDNA sequences, each one tagged with the description of a corresponding library. We developed software for EST sorting on the basis of the cDNA tissue source. It enabled us to find a set of genes highly expressed in different tumours but not in normal tissues. The products of such genes could serve as novel tumour markers and potential targets for anti-tumour therapy.

2. Materials and methods

The digital differential display (DDD) tool located on the UNIGENE site was used to perform a model subtraction of libraries available on the same site (http://www.ncbi.nlm.nih.gov/UniGene/). The human section of the dbEST database was obtained from the publicly available NIH site (http://www.ncbi.nlm.nih.gov). EST clusters containing cDNA-derived sequences of the same transcribed unit were obtained from UNIGENE Build 129 which contained the following data: EST descriptions including library identifiers as well as the nucleotide sequences and indexes of all ESTs; ESTs grouped in about 90 000 clusters by internal algorithms developed by NCBI (see the description at http://www.ncbi.nlm.nih.gov/UniGene/build.html); and short descriptions of all libraries contributing to the database.

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Table 1
Tumour-specific ESTs found by DDD tool

No.	UniGene ID	Protein function (if known) or closest homologue	Protein homology, %/length, aa	Tumour-derived ESTs/total ESTs	Types of tumours	Normal tissues
1	Hs.30868	protein weakly similar to human GPV platelet glycoprotein V precursor	46%, 55 aa	23/30	ovarian carcinoma, lymphoma, germ cell tumour, uterine carcinoma, breast carcinoma, Wilms tumour, leiomyosarcoma, lung carcinoma, HNSCC	total foetus, infant brain, pooled tissues
2	Hs.86232	protein similar to growth differentiation factor 3 (mouse)	84%, 96 aa	29/30	germ cell tumours	brain, kidney
3	Hs.89436	cadherin 17 (liver-intestine)	100%, 831 aa	46/52	colon carcinoma, pancreatic adenocarcinoma, stomach carcinoma	colon
4	Hs.98517	ESTs		20/23	ovarian tumour, germ cell tumour, melanoma, PNET	infant brain, adult brain, pooled tissues
5	Hs.104134	homeobox (H6 family) 1 protein	100%, 372 aa	18/20	anaplastic oligodendroglioma, lung carcinoma, medulloblastoma	pooled tissues
6	Hs.105484	protein weakly similar to human lithostahine 1 β precursor	35%, 130 aa	100/105	pancreatic carcinoma, colon carcinoma, lung carcinoma, stomach carcinoma, breast carcinoma, ovarian carcinoma, CLL, germ cell tumour, AML	pooled foetal tissues, colon
7	Hs.111939	*Homo sapiens* cDNA FLJ20470 fis, clone KAT06815	–	25/28	glioblastoma, ovarian tumour, colon carcinoma, lung carcinoma, breast carcinoma, gastric tumours, anaplastic oligodendroglioma, medulloblastoma, choriocarcinoma	schizophrenic brain, nervous tissues
8	Hs.114905	protein homologous to IRE1 (*Saccharomyces cerevisiae*)	38%, 128 aa	20/21	colon carcinoma, pancreatic carcinoma, gastric tumour, stomach carcinoma, pancreatic adenocarcinoma	colon
9	Hs.115947	keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	100%, 472 aa	44/54	HNSCC, endometrial carcinoma, ovarian carcinoma, cervical tumour, uterine tumour, germ cell tumour, bladder tumour, laryngeal SCC, lung carcinoma, pancreatic carcinoma, oral SCC, neuroblastoma, esophageal SCC	normal prostate epithelium cell culture, human epidermal keratinocytes, mammary gland, pooled tissues, placenta
10	Hs.116051	protein highly similar to OCIM (oncogene in multiple myeloma) protein	90%, 344 aa	39/45	Wilms tumour, CLL, colon carcinoma, germ cell tumour, clear cell kidney tumour, lung carcinoma, ovarian fibrothecoma and carcinoma, pancreas carcinoma, neuroblastoma, choriocarcinoma, gastric carcinoma	breast, foetal heart
11	Hs.127383	ESTs		17/18	colon carcinoma	pooled tissues
12	Hs.129302	ESTs		24/26	germ cell tumour, lung carcinoma	pooled tissues
13	Hs.132816	ESTs		26/33	colon carcinoma, germ cell tumours, laryngeal SCC, lung carcinoma, bladder carcinoma, lung carcinima	prostate, pooled tissues, testis
14	Hs.133294	protein weakly similar to human RAS GTPase-activating-like protein IQGAP1	61%, 124 aa	58/63	prostate tumour, HNSCC, breast carcinoma, oligodendroglioma, colon carcinoma, CML, lung carcinoma, ovarian carcinoma, uterus carcinoma, adrenal adenoma	pooled tissues
15	Hs.133300	ESTs		47/50	breast carcinoma, oligo dendroglioma, lung carcinoma, ovarian carcinoma	pooled tissues
16	Hs.134012	C1q-related factor	100%, 257 aa	39/46	glioblastoma, oligodendroglioma, medulloblastoma, glioblastoma, germ cell tumour, kidney tumour, placenta choriocarcinoma, uterine carcinoma, uterine leiomyosarcoma, lung carcinoma, ovarian carcinoma, oligodendroglioma	pooled tissues, prostate
17	Hs.137031			25/31	germ cell tumours	pooled tissues, prostate
18	Hs.144121	protein weakly similar to dJ37E16.2	34%, 118 aa	25/27	glioblastoma, oligodendroglioma, lung carcinoma	adult brain, colonic mucosa
19	Hs.145508	ESTs		33/36	lung and ovarian carcinomas	pooled libraries
20	Hs.157205	branched chain aminotransferase 1, cytosolic	100%, 383 aa	32/36	oligodendroglioma, germ cell tumours, ovarian and lung carcinomas, rhabdomyosarcoma, lung small cell carcinoma, terato-carcinoma, uterus tumour	germinal centre B cells, pooled libraries

Table 1 (*continued*)

No.	UniGene ID	Protein function (if known) or closest homologue	Protein homology, %/length, aa	Tumour-derived ESTs/total ESTs	Types of tumours	Normal tissues
21	Hs.172632	ESTs		17/18	breast carcinoma, oligodendroglioma, ovarian carcinoma	normal bone marrow
22	Hs.175190	ESTs		35/39	breast, lung and ovarian carcinoma, oligodendroglioma	breast epithelium
23	Hs.133107	ESTs		20/20	ovarian and lung carcinomas, oligodendroglioma	
24	Hs.133296	ESTs		18/18	ovarian and lung carcinomas	
25	Hs.145492	ESTs		16/16	ovarian and lung carcinomas	
26	Hs.181624	ESTs		78/78	ovarian and lung carcinomas	

AML, acute myelogenous leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myelogenous leukaemia; HNCCS, head and neck squamous cell carcinoma; PNET, peripheral primitive neuroectodermal tumour; SCC, squamous cell carcinoma.

These files are available at (ftp://ncbi.nlm.nih.gov/repository/UniGene/). Any EST cluster linked to the descriptions of all incorporated ESTs can be obtained using UNIGENE search engine (http://www.ncbi.nlm.nih.gov/UniGene/).

We have developed a program called HSAnalyst to classify data from original dbEST and UNIGENE databases in a table form. The program is available at (http://pcn197.vigg.ru/programs/HSAnalyst.exe). This program works with a supplementary database called LibraryRegistry which includes entries describing all human cDNA libraries classified by tissue source. This database contains a description of libraries cross-referenced from different data sources including dbEST, UNIGENE, CGAP (web-site available at NIH site), TIGR (www.tigr.org) and Stratagene (www.stratagene.com). HSAnalyst software is able to arrange EST data according to any given parameter, e.g. tissue type or the number of ESTs contained in a cluster.

Standard BLAST and FASTA program families were used to analyse the clusters of interest.

3. Results and discussion

We have carefully checked the descriptions of every library we used to ensure that no libraries and, consequently, cDNA clones were misclassified as 'normal' or 'tumour'. Using available sources including UNIGENE, dbEST, TIGR, STRATAGENE, we have created the LibraryRegistry database containing verified descriptions of all cDNA libraries. In this database, libraries are classified as 'tumour' or 'normal' according to their origin from tumour or normal tissues (cells). Only well characterised libraries have been included in the database. Libraries classified as 'premalignant', 'non-cancerous pathology' and 'immortalised cells' have not been included. Some libraries were rated as undefined due to lack of data or ambiguous information, and were not included. This work has revealed many mistakes and data losses in the original library descriptions. In total, 2681 libraries were classified as 'tumour' and 1087 libraries as 'normal'. The resulting database contains 921 237 'tumour' ESTs incorporated in 56 241 clusters and 810 097 'normal' ESTs incorporated in 53 762 clusters. 500 462 ESTs could not be classified as 'tumour' or 'normal' libraries. The 'normal' subset was subtracted from the 'tumour' in silico resulting in a group of genes highly expressed in tumour, but not in normal tissues.

As a pilot phase of the experiment we chose all libraries derived from tumour tissues, and performed the DDD procedure versus the set of libraries derived from normal tissues. This work was performed in January 2001. At this time the DDD script operated with about 25% of total libraries from the publicly available dbEST database. As a result, we have obtained 41 clusters satisfying the following criteria: (1) each cluster contains more than 10 ESTs belonging to a unique cluster and (2) each cluster contains not more than one EST from normal libraries. After the UNIGENE database was rebuilt some clusters either changed dramatically or were totally eliminated from the database due to new alignment results. Only 26 of 41 clusters preserved their structures, anchoring ESTs and tumour EST prevalence after four successive UniGene builds. These clusters were considered stable. However, 22 of 26 clusters ceased fitting the criterion (2) as they pumped in normal library-derived ESTs during the subsequent growth of the dbEST database. All 26 stable clusters were analysed by means of BLAST and FASTA tools and compiled in Table 1. Only six of the 26 clusters listed in Table 1 correspond to human genes with well-established functions. Most tumour-specific clusters obtained by this method were considered 'new' genes because they have not been found to

Table 2
Tumour-related clusters at different ranges

Range	EST number		Tumour-specific EST (%)	Number of tumour-related clusters at threshold (%)[a]			
	entries	clusters		>90%		100%	
				observed	expected	observed	expected
1–2	59 111	44 373	42	18 342	23 073	18 342	23 073
3–4	45 400	13 401	35	1880	1884	1880	1884
5–8	53 569	8742	37	567	279	567	172
9–16	63 421	5407	39	168	5	99	4
17–32	83 968	3607	41	**45**	**0**	**17**	**0**
33–64	176 845	3762	43	**16**	**0**	**2**	**0**
65–128	349 008	3790	45	**10**	**0**	**2**	**0**
129–256	460 493	2588	47	**8**	**0**	**0**	**0**
257–512	339 482	975	50	**3**	NA[b]	**0**	NA
513–1024	208 171	303	53	**1**	NA	**0**	NA
1025–2048	130 524	96	57	0	NA	0	NA
2049–4096	95 180	36	60	0	NA	0	NA
4097–8192	49 804	10	66	0	NA	0	NA
8193–16 384	14 725	1	67	0	NA	0	NA

The cases where the calculated number of clusters is less than one are shown in bold.
[a]Expressed as the persentage of tumour-specific EST in the clusters.
[b]NA, not applicable. The ranges over 257 were not computed due to the lack of significant digits.

have any defined function in the human organism. Eight of the 20 remaining EST clusters represent genes displaying a homology to known eucaryotic proteins, and 12 represent unknown expressed sequences.

A program named HSAnalyst was developed to conduct global analysis of cluster data. For complete CDD analysis we used the LibraryRegistry database consisting of about 4000 library descriptions (this number is continually increasing with the addition of new entries). An algorithm executed by the program consists of two major steps: (1) for each cluster the number of ESTs is retrieved from the cluster description and (2) the number of ESTs from the 'tumour' cDNA libraries is counted according to the LibraryRegistry database. The whole range of possible EST numbers is dissected into sub-ranges. HSAnalyst makes it possible to arrange sub-ranges exponentially (sub-ranges with exponents 1–2, 3–4, 5–8, 9–16, etc.) or linearly (sub-ranges with factors 1–10, 11–20, 21–30, etc.). Simultaneously, the ratio cancer ESTs/all ESTs is calculated for each cluster, and those which exceed the user-defined bottom threshold value are listed in the output file. To be sure that we found 'true' tumour-specific clusters not generated by chance among the total number of the EST clusters (more than 90 000 units) we calculated the theoretical number of 'tumour' clusters for every sub-range. The underlying model was the binomial distribution with the mean value of 'cancer/all' ratio that could be defined by a user (0–100%). The number of clusters that exceeded threshold value was calculated.

HSAnalyst output data describing two sets of clusters containing more than 90 and 100% of tumour-related ESTs are shown in Table 2. For each range of cluster sizes, 'cancer-related' ESTs content, the number of clusters that comply with the input conditions and the expected number of such clusters are calculated. For the current database, the mean content of 'cancer-related' ESTs is about 48%. Table 2 illustrates that tumour-specific clusters are not accidental but represent a natural phenomenon. The results found by HSAnalyst agree with the data presented in Table 1: all 26 clusters enlisted in Table 1 remain constant during UniGene database growth. All of them are within the range of 17–128 by the number of ESTs included in a cluster (counted with a threshold >90%). Twenty-one clusters falling in the range of 17–128 contain only tumour-derived ESTs and fulfil the 100% threshold. Four of them coincide with strictly tumour-specific stable clusters found by DDD search, presented in Table 1 (NN 23–26). Seventeen extra clusters found during the global analysis of cluster data and representing exclusively tumour-derived mRNAs found by the HSAnalyst tool are presented in Table 3.

All genes or gene fragments presented in Tables 1 and 3 have displayed high expression level in tumours and significantly lower expression level in normal tissues. Both Tables 1 and 3 contain clusters (genes) that have high similarity or significant homology to known proteins and simultaneously have a high content of tumour ESTs. Table 1 includes two genes which encode proteins involved in cell contacts (liver–intestine cadherin (LI) and keratin 16, mutated in focal non-epidermolytic palmoplantar keratoderma), C1 complement-related factor, homeo box protein 1 belonging to H6 family, and cytosolic branched chain aminotransferase 1. Among genes overexpressed in tumour tissues containing open reading frames with significant homologies to the known proteins, the most interesting are those related to proteins involved in carcinogenesis. They include a close homologue of an oncogenic protein OCIM (90% of 344 amino acids) which plays a role in multiple myeloma development; a homologue of mouse growth differentiation factor 3 (84% of 96 amino acids); and a protein with weak homology to IQGA human RAS GTPase-activating-like protein IQGAP1. Possible involvement of these genes in human cancer development warrants further investigation.

The most interesting clusters were represented by ESTs found exclusively in the tumour-derived libraries. Four tumour-specific clusters are represented in Table 1 (NN 23–26) and 17 more in Table 3. The striking feature of the analysed tumour-specific clusters is their frequent occurrence in libraries from colon carcinomas (Hs.560, Hs.1085, Hs.239891) or lung and ovarian carcinomas (Hs.145340, Hs.145509, Hs.181624, Hs.293429, Hs.133107, Hs.133296, Hs.145492, Hs.181624). Interestingly, all three colon-specific EST clusters

Table 3
Tumour-specific ESTs found by global analysis of cluster data

No.	UniGene cluster ID	Gene name and function (if known)	Protein homology, %/length, aa	Tumour-derived ESTs/total ESTs	Types of tumours
1	Hs.560	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	100%/235 aa	55/55	colon carcinoma, CLL
2	Hs.1085	guanylate cyclase 2C (heat stable enterotoxin receptor)	100%/1072 aa	17/17	colon carcinoma
3	Hs.67624	ESTs		19/19	germ cell tumour, HNSCC
4	Hs.145340	ESTs		21/21	lung carcinoma, ovary carcinoma, HNSCC
5	Hs.145509	ESTs		24/24	breast carcinoma, lung carcinoma, ovary carcinoma, stomach carcinoma, oligodendroglioma
6	Hs.152531	heart and neural crest derivatives expressed 1	100%/214 aa	18/18	germ cell tumour, pooled sarcomas, Schwannoma, neuroblastoma
7	Hs.154173	ESTs		23/23	lung carcinoma, testicular teratocarcinoma
8	Hs.156810	ESTs		27/27	brain tumours (meningioma, oligodendroglioma, medulloblastoma, astrocytoma), germ cell tumour, stomach carcinoma, pancreatic carcinoma, colon carcinoma, lymphoma, endometrial carcinoma, rhabdomyosarcoma
9	Hs.172708	ESTs		23/23	ovariam carcinoma, breast carcinoma, oligodendroglioma
10	Hs.181624	ESTs		78/78	breast, lung and ovarian carcinomas
11	Hs.196073	ESTs		20/20	germ cell tumour, genito-urinary tract transitional cell tumours, lung carcinoma, stomach carcinoma
12	Hs.239891	G protein-coupled receptor 35	100%/308 aa	33/33	colon carcinoma
13	Hs.253298	ESTs		72/72	germ cell tumour, HNSCC
14	Hs.272216	glycoprotein VI (platelet)	100%/338 aa	20/20	colon carcinoma, epididimal tumour, HNSCC, rhabdomyosarcoma, neural tumours
15	Hs.279805	ESTs		18/18	lung carcinoma, neural tumours, PNET
16	Hs.285026	HERV-H LTR-associating protein 1	100%/387 aa	22/22	colon carcinoma, germ cell tumour, HNSCC, AML
17	Hs.293429	ESTs		30/30	breast, lung and ovarian carcinomas

AML, acute myelogenous leukaemia; CLL, chronic lymphocytic leukaemia; HNCCS, head and neck squamous cell carcinoma; PNET, peripheral primitive neuroectodermal tumour.

obtained by our analysis represent the known genes encoding apolipoprotein B mRNA-editing protein APOBEC1, guanylate cyclase 2C and G protein-coupled receptor 35. Both APOBEC1 and guanylate cyclase 2C mRNAs have been shown to be overexpressed in colon carcinomas [7,8]. Moreover, the high-level expression of APOBEC1 in transgenic mice and rabbit liver causes liver dysplasia and hepatocellular carcinomas [9]. mRNA encoding guanylate cyclase 2C appears to be a relatively specific marker for the presence of metastatic colonic carcinoma cells in normal tissues including peripheral blood [8]. In our opinion, the gene encoding G protein-coupled receptor 35 deserves attention as a putative marker of colon cancer possibly involved in the progression of this important disease.

EST clusters from lung and ovarian carcinoma libraries may also represent potential tumour markers. These clusters do not contain any homologies to the known proteins and open reading frames easily recognised. They may be considered evidence in favour of the expression of newly evolved DNA sequences in tumour cells [5] or as a manifestation of 'background' or 'illegitimate' gene expression [10,11] which may be enhanced in tumour cells due to dysregulation of the house-keeping processes.

Differentially expressed EST clusters (genes) can be useful as tumour markers and prognostic indicators and can be suitable targets for various therapeutic interventions. We probed a subset of EST clusters presented in Table 1 (six out of 26) and Table 3 (five out of 17) by confirmatory polymerase chain reaction experiments on Clontech multiple tissues cDNA (MTC) panels. The results are reassuring as mRNA corresponding to the probed clusters Hs.133294, Hs.279805, Hs.196073, Hs.154173, Hs.145340 and Hs.67624 showed expression in different tumours but not in normal tissues included in MTC panels (data not shown). This confirms the validity of the CDD procedure applied in this article.

Patent application (disclosed) filed.

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