A profile of differentially expressed genes in primary colorectal cancer using suppression subtractive hybridization

Simon E. Hufton[a,c], Peter T. Moerkerk[b], Ricardo Brandwijk[b], Adriaan P. de Bruïne[a], Jan-Willem Arends[a,b], Hennie R. Hoogenboom[b,c,*]

[a] *Research Institute Growth and Development, Department of Pathology, University Hospital Maastricht, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands*
[b] *Maastricht University, Maastricht, The Netherlands*
[c] *Target Quest B.V., Maastricht, The Netherlands*

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Abstract As a step towards understanding the complex differences between normal cells and cancer cells, we have used suppression subtractive hybridization (SSH) to generate a profile of genes overexpressed in primary colorectal cancer (CRC). From a 35 000 clone SSH-cDNA repertoire, we have screened 400 random clones by reverse Northern blotting, of which 45 clones were scored as overexpressed in tumor compared to matched normal mucosa. Sequencing showed 37 different genes and of these, 16 genes corresponded to known genes in the public databases. Twelve genes, including Smad5 and Fls353, have previously been shown to be overexpressed in CRC. A series of known genes which have not previously been reported to be overexpressed in cancer were also recovered: Hsc70, PBEF, ribophorin II and Ese-3B. The remaining 21 genes have as yet no functional annotation. These results show that SSH in conjunction with high throughput screening provides a very efficient means to produce a broad profile of genes differentially expressed in cancer. Some of the genes identified may provide novel points of therapeutic intervention.

Key words: Colorectal cancer; Gene expression; Suppression subtractive hybridization

1. Introduction

The formation and progression of tumors are associated with changes in protein expression, which in most cases can be correlated with abnormal levels of mRNA. The mortality from colorectal cancer (CRC) has changed very little over the past 50 years, which emphasizes the need for therapies based on a greater understanding of the molecular changes that underlie CRC. The value in the identification of overexpressed molecules in CRC is three fold: (i) overexpressed genes may be used as markers for biological behavior such as invasiveness or metastasis, which could be of significant value for prognosis and therapeutic management, (ii) differentially expressed tumor cell surface markers may be targets for antibody-based immunotherapy [1], (iii) understanding the phenotypic changes associated with the overexpression of cancer genes allows for the formulation of pathways or targets for therapeutic intervention.

There is clearly a need for methods that allow for the rapid identification of differentially expressed genes associated with a particular disease state. The first approach to describe the identification of differentially expressed genes was based on subtractive hybridization. This method was eventually superseded by differential display [2]. The development of serial analysis of gene expression (SAGE) [3,4] and cDNA microarray technology [5,6] has allowed for the generation of global expression profiles in cancer. Although both SAGE and cDNA microarrays have proved powerful tools for the construction of gene indexes, they have a critical limitation in that both methods are dependent on the availability of previously cloned genes.

We have used an alternative method called suppression subtractive hybridization (SSH) [7] because this method is not dependent on the availability of previously cloned cDNA sets and will allow for the cloning of informative fragments of unknown gene sequences. SSH allows for the normalization of frequent and rare cDNAs and subtraction of cDNAs common between two populations. Suppression PCR allows for the exponential amplification of differentially expressed genes and the suppression of sequences present in equal amounts in both cDNA populations. This technique is capable of high enrichment factors [7] and is amenable to microarray analysis. Arrayed SSH repertoires will have the advantage that redundant spotting will be eliminated and much smaller and efficient DNA chips can be produced [8].

We have used SSH for the first time to identify differentially expressed genes in primary CRC. We have generated a large repertoire which is highly enriched for overexpressed genes. From this repertoire, we have isolated many novel gene sequences of which epithelial specific Ets transcription factor 3B (Ese-3B), pre-B cell enhancing factor (PBEF), heat shock cognate 70 (Hsc70), ribophorin II and 21 yet to be functionally annotated genes are of particular interest.

2. Materials and methods

2.1. Tumor material

Fresh frozen tissue from a primary stage III rectal adenocarcinoma and matched upstream non-neoplastic mucosa were obtained from a 60 year old Caucasian male following surgical resection. This tissue was used for cDNA library construction.

2.2. mRNA isolation and cDNA synthesis

Total RNA was isolated from both normal mucosa and tumor using a RNA extraction kit (QIAGEN). We purified mRNA using

*Corresponding author. Fax: (31)-43-387 6613.
E-mail: hho@lpat.azm.nl

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oligotex beads (QIAGEN). Using 0.5 μg mRNA, cDNA synthesis was performed using the Capfinder cDNA synthesis kit according to the manufacturer's instructions (Clontech).

2.3. Generation of cDNA library using SSH

SSH was performed between primary colorectal tumor cDNA and matched normal mucosal cDNA, using the PCR select cDNA subtraction kit (Clontech) according to the manufacturer's recommendations.

2.4. Cloning of the SSH repertoire into a T/A cloning vector

The SSH-cDNA repertoire was cloned into the T/A cloning vector pCR2.1 (Invitrogen) and transformed into the *Escherichia coli* strain TG1 by electroporation (Bio-Rad) at 2.5 kV, 25 μF and 200 Ω. The library was plated on 2×TY agar plates (16 g/l bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl, 15 g/l bacto-agar) containing ampicillin at 100 μg/ml and 2% w/v glucose. After overnight growth at 37°C, the repertoire was recovered in 2×TY medium plus ampicillin at 100 μg/ml by flooding the plates and frozen in aliquots in 15% w/v glycerol. The size of the cDNA library was determined by plating an original sample on 2×TY plates containing ampicillin, X-gal (40 μl of a 40 mg/ml stock per 90 cm plate) and IPTG (40 μl of a 100 mM stock per 90 cm plate).

2.5. Screening using reverse Northern blotting

Individual recombinant clones were inoculated into sterile 96 well microtiter plates (Costar) containing 100 μl of 2×TY medium, containing ampicillin at 100 μg/ml, and were grown overnight at 30°C, after which 5 μl was removed and transferred to PCR 96 well tubes (Sarstedt). PCR was performed and inserts were amplified using specific primers (Invitrogen).

After PCR, 10 μl samples were loaded onto 1.5% agarose TBE gels (Hybaid) in duplicate (10 μl per slot with 80 samples on each gel). PCR products were denatured in 0.4 M NaOH, 0.6 M NaCl and blotted onto nylon membranes (Schleicher and Schuell). After transfer, blots were neutralized using 2×SSC (0.3 M NaCl, 30 mM sodium citrate pH 7.0), 0.5 M Tris-HCl pH 7.4. Filters were hybridized as in [9] and probed with equivalent amounts of ^{32}P-labelled (Boehringer Mannheim), *Rsa*I-digested double-stranded cDNA, derived from normal mucosa and CRC cDNA. Both probes were of approximately equal specific activity (10^9 dpm/μg DNA). Filters were washed with 0.5×SSC, 1% SDS at room temperature. Duplicate filters were prepared and differential signals between colon tumor cDNA fragments and normal cDNA fragments identified after overnight exposure to a phosphorimaging screen (Bio-Rad). Densitometric scanning of duplicate blots allowed for the calculation of the ratio of signal obtained with tumor to normal cDNA probes and normal cDNA probes. Positive clones were sequenced and submitted to the National Centre for Biotechnology Information for homology search.

2.6. Northern analysis and semi-quantitative reverse transcriptase (RT-) PCR

Total RNA was prepared and analyzed as in [9]. Probes were generated by PCR amplification and labelled using random priming (Boehringer Mannheim). Unincorporated label was removed prior to hybridization using Sephadex G50. Filters were then exposed to a phosphorimaging screen (Bio-Rad).

RNA was normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by Northern blotting and first strand cDNA synthesis was performed using random primers. As a control for genomic DNA contamination, all reactions were set up in duplicate with the control sample lacking reverse transcriptase. Gene specific primers were designed to amplify fragments between 400 and 700 bp during 39 cycles at an annealing temperature of 55°C. Primers for c-Myc are coding strand primer (5′-AAGCTCGTCTCAGAGAAGCT-3′), non-coding strand primer (5′-AGCCTGCCTCTTTTCCACAG-3′); for secreted protein, acidic and rich in cysteine (SPARC) coding strand primer (5′-CCATGGAGCATTGCACCACCCGC-3′), non-coding strand primer is (5′-CAGATCCGTGTCCACCCATGTGCC-3′); for interferon inducible protein 1-8D (IFN 1-8D) coding stand primer is (5′-GCCATTGTAGAAAAGCGTGTGAGG-3′), non-coding strand primer is (5′-CGCCTACTCCGTGAAGTCTAGGG-3′); for PBEF coding strand primer is (5′-GTGCCTGTATCTGTGGTCAG-3′), non-coding strand primer is (5′-CACACACCCAGTCATAAAGCC-3′). Ten μl aliquots were removed from the PCR reaction at 20, 24, 28, 32 and 36 cycles and ran on a 1.5% TBE agarose gel. Band intensities were compared between matched samples of tumor and normal mucosa, GAPDH expression was included as an internal control to normalize the differential signal ratio between normal and tumor samples.

2.7. In situ hybridization

To test expression using in situ hybridization, SPARC was cloned into pCR2.1 in two orientations. Antisense and sense RNA probes were generated by T7 RNA polymerase using digoxigenin (DIG)-labelled UTP (Roche Diagnostics). Five μm tissue sections of both neoplastic and normal colon were hybridized with 100 ng/ml antisense DIG-RNA and sense DIG-RNA as a negative control. After incubation with anti-DIG antibodies labelled with alkaline-phosphatase, the probes were visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as in [10].

3. Results

3.1. Identification of genes overexpressed in colon cancer using SSH

As previous results using SSH have concentrated on using cell lines [11], we decided to apply SSH to a single matched tumor normal tissue pair. Inserts from 400 random clones from a 35 000 independent clone SSH repertoire were amplified by PCR and then separated on a gel for subsequent reverse Northern analysis. This gave clear hybridization signals and genes differentially expressed between normal mucosa and tumor could be easily detected (Fig. 1). The differential signal obtained by reverse Northern blotting was quantitated using a phosphorimager and the average increase in signal in tumor compared to normal was approximately six for genes described in both Table 1 and Table 2. Of the 400 clones analyzed, 45 were detected as overexpressed in tumor compared to normal and 63 genes gave a positive hybridization signal but did not show overexpression in tumor material. The remaining 292 genes gave no hybridization signal. Of these, we expect approximately 41% to be differentially expressed but lying outside the sensitivity limit of the reverse Northern analysis. These probably represent the 'rarest' transcripts. The total number of genes analyzed that showed a differential signal was 11%. This is in close agreement with that previously reported by Von Stein et al. (1997) [11], who reported that 12.5% of genes recovered by SSH showed a differential signal using a similar screening assay.

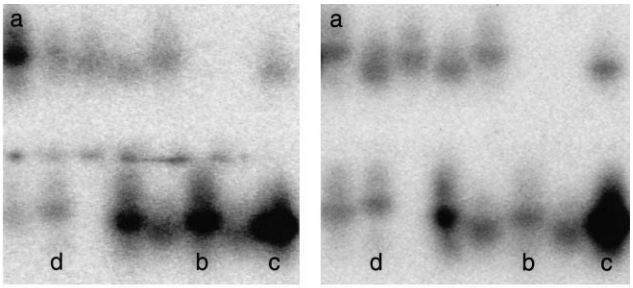

Fig. 1. An example of a reverse Northern blot. Example of part of a reverse Northern blot probed with complex cDNA probes prepared from primary tumor tissue and normal mucosa. Bands a and b represent genes overexpressed in the tumor, c represents a gene showing equal abundance in both normal and tumor tissue, d represents background binding and thus remains undetected by reverse Northern screening.

Of the 45 genes identified as overexpressed, 37 represented different transcripts and of these, 16 showed homology to known genes in the public databases. Furthermore, 12 genes have previously been shown to be overexpressed in cancer: CEA [12], NCA [13], c-Myc [14], SPARC [15], fibronectin precursor [16], cytochrome *c*-oxidase subunit I, ATPase-6 [17], lactate dehydrogenase B (LDH-B), [18], IFN 1-8D [4] Caldesmon [19], Smad5 [20] and Fls353 (GenBank submission March 1999, accession number AB024704) (Table 1). The remaining 21 genes showed no significant homology with any functionally annotated sequences in the public databases (Table 2). It is interesting to note that our SSH repertoire had a surprisingly low level of redundancy in that of the 45 genes identified, only five genes (CEA, NCA, Smad5, cytochrome *c*-oxidase subunit I and ATPase-6) were recovered more than once.

The average fragment insert size corresponding to the known genes was 723 bp, which is substantially larger than the 256 bp that can be statistically predicted by the 4 bp restriction enzyme used to generate the cDNA fragments for SSH. This has been reported previously and is due to the suppression PCR effect being more efficient for shorter molecules of less than 200 nucleotides [7]. This preferential enrichment of longer molecules is balanced by the tendency of the subtraction procedure to favor shorter cDNA fragments which are more efficiently hybridized, amplified and cloned than longer fragments.

3.2. *Confirmation of overexpression of selected genes by a second independent method*

For further target validation, we have used semi-quantitative RT-PCR, Northern analysis and in situ hybridization. We have confirmed overexpression of c-Myc, SPARC and PBEF in three matched tumor normal samples by semi-quantitative RT-PCR (Fig. 2) of which PBEF has not previously been reported as overexpressed in CRC. Further confirmation of overexpression of SPARC was provided by in situ hybridization and preferential staining of the vessel wall in tumor tissue compared to matched normal mucosa can be seen (Fig. 3). Using Northern analysis, we have confirmed IFN 1-8D and

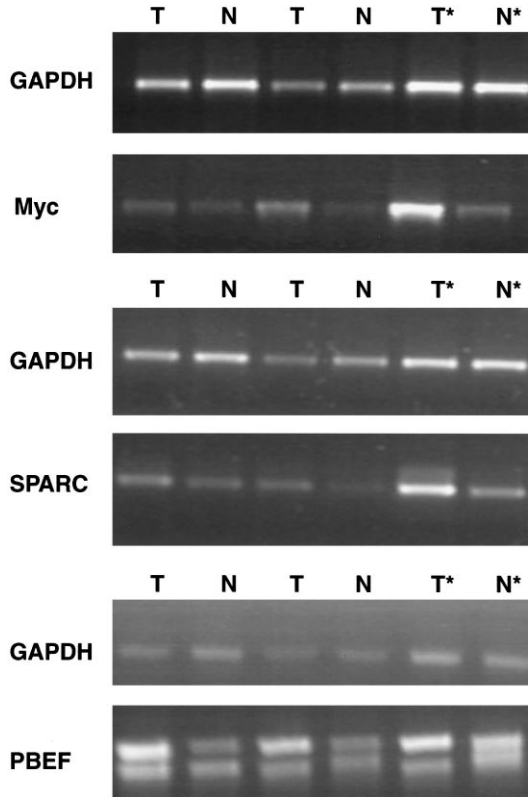

Fig. 2. Semi-quantitative RT-PCR of selected overexpressed genes. The figure shows semi-quantitative RT-PCR for matched tumor and normal mucosa samples. Samples were normalized using the housekeeping gene GAPDH which did not show a differential signal between tumor and normal tissue. Analysis was performed on the known genes SPARC, IFN 1-8D, c-Myc and PBEF. Matched tissue samples used to construct the SSH library are indicated with an asterisk.

Table 1
List of overexpressed genes in primary CRC with homology to known sequences in the public databases

Identity	Homology[a]	Ratio T/N[b]	Appearance[c]	Accession no.[d]
Col-p1	Cytochrome *c*-oxidase 1	6	2	J01415
Col-p2	CEA	7.5	3	M17303
Col-p3	NCA	7.8	4	M18728
Col-p4	SPARC	2.4	1	M25746
Col-p7	c-Myc	7	1	J00120
Col-p11	Ribophorin II	4.5	1	NM002951
Col-p13	Fibronectin precursor	10	1	K00799
Col-p15	Ese-3B	5.6	1	AF124439
Col-p16	Hsc70	7.9	1	Y00371
Col-p19	Smad5	3.4	2	U59913
Col-p29	ATPase subunit-6	9	2	J01415
Col-p35	Caldesmon 3′UTR	7.25	1	X93334
Col-p37	PBEF	6	1	U02020
Col-p38	LDH-B	10.5	1	Y00711
Col-p42	IFN 1-8D	6	1	X57351
Col-p49	Fls353	3.0	1	AB024704

[a]Gene with functional annotation with which maximum homology is obtained using a BLAST search of the public databases.
[b]The increase in signal obtained with the complex tumor cDNA probe compared to the complex normal cDNA probe as obtained by reverse Northern analysis and quantitated using densitometric scanning.
[c]The number of times that the known gene appears in the analysis of 400 clones.
[d]The accession number in the public databases.

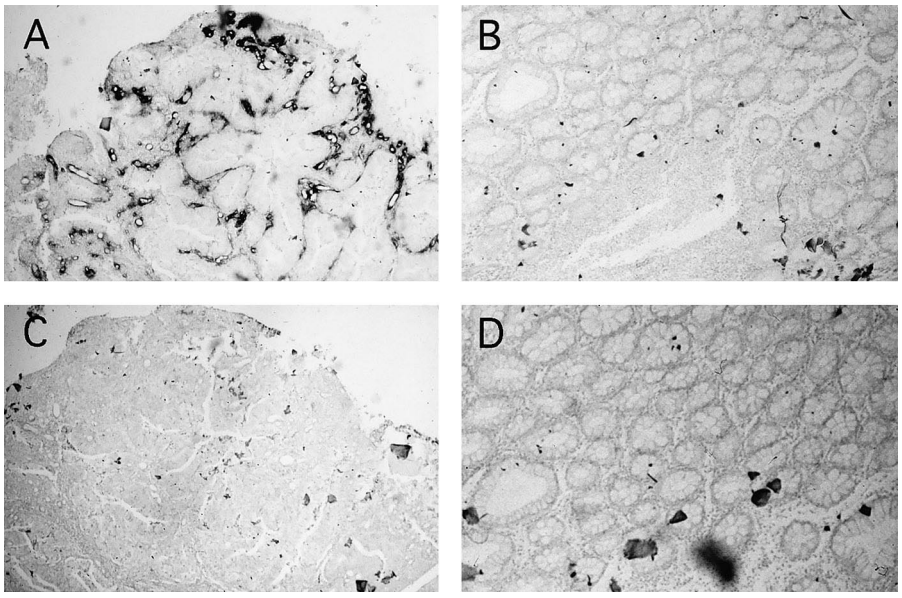

Fig. 3. Detection of SPARC expression by in situ hybridization. A: Colorectal tumor tissue probed with a labelled SPARC antisense RNA. B: Normal mucosa probed with labelled SPARC antisense RNA. C: Colorectal tumor tissue probed with labelled SPARC sense RNA. D: Normal mucosa probed with labelled SPARC sense RNA.

LDH-B in $n = 3/4$ and $n = 4/4$ matched tumor and normal samples, respectively (Fig. 4).

4. Discussion

We have generated for the first time a large collection of differentially expressed genes in primary CRC by reverse Northern screening of a SSH-derived cDNA library. We decided to generate a profile of differentially expressed genes in primary tumor tissue rather than in tumor cell lines, believing that this will give a more physiologically relevant output of abnormally regulated genes. Although cell lines are an extremely useful and consistent source of tumor material, a cautionary note has most recently been provided by the SAGE method. Only 26% of the genes identified as overexpressed in

Table 2
Overexpressed genes in primary CRC with no functional annotation

Identity	Ratio T/N[a]	Accession no.[b]
Col-p8	6	AA455483
Col-p9	4.8	AA449997
Col-p12	5.4	No homology
Col-p14	5.3	U75653
Col-p17	5.3	AA535206
Col-p18	5.7	AA635946
Col-p20	15.6	AA252109
Col-p22	3.5	AA782573
Col-p23	4.2	AA236320
Col-p24	9.6	No homology
Col-p25	10.8	AA313600
Col-p26	5.4	N33897
Col-p27[c]	17	D87666
Col-p28	2.6	N44337
Col-p30	4.6	AI142134
Col-p39	3.8	AA336609
Col-p40	5.6	AI188015
Col-p43	6.3	AA314633
Col-p44	5.3	W07312
Col-p45	5	AA040810
Col-p50	14	AA029988

[a]Quantitated increase in signal obtained with the tumor complex cDNA probe compared to the complex normal cDNA probe in reverse Northern analysis.
[b]The accession number in the public databases.
[c]Col-p27 has homology to a Hsp90-related protein. All gene fragments shown were recovered once in the reverse Northern screening analysis.

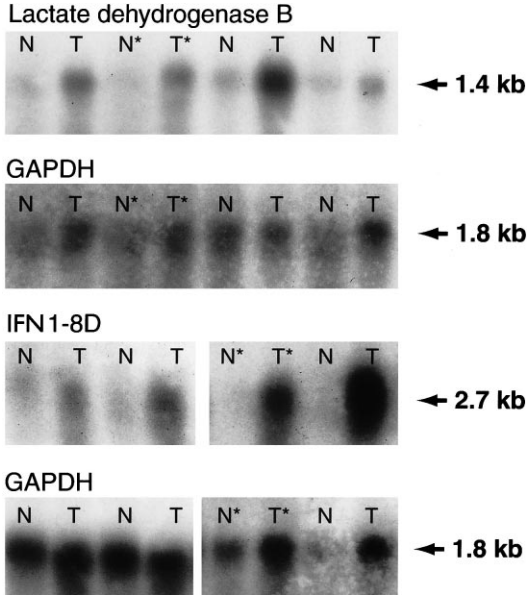

Fig. 4. Northern analysis of selected overexpressed genes. Northern analysis of matched primary tumor and normal total RNA samples. Total RNA samples were normalized for GAPDH prior to loading. LDH-B gave a band of the expected size (1.4 kb) and showed overexpression in tumor compared to normal in four matched samples. IFN 1-8D which is only a partial gene sequence in the public database gave a band of 2.7 kb in Northern blotting and showed overexpression in tumor compared to normal in three out of four matched samples. The tumor normal sample pair used to construct the SSH library is shown by an asterisk.

colon cancer cell lines were also shown to be overexpressed in primary tumor tissue [4]. Most of the known genes we have recovered have not been reported by the SAGE method with only four genes being common to both our SSH analysis and SAGE (i.e. fibronectin precursor, SPARC, LDH-B and IFN 1-8D). However, the vast majority of known genes we have recovered by SSH have previously been correlated with cancer using other techniques. This suggests that although there is overlap in the profile of genes overexpressed, there may be different biases associated with each technique.

The SSH procedure is ideally suited for the identification of oncogenes. One of the new genes we have recovered is Col-p15, which has recently been described as Ese-3B (accession number AF124439). Ese-3B is a new member of the Ets transcription factor family which includes several oncogenes that induce tumorigenesis when overexpressed. The Ese-3B protein is most closely related to the recently identified ESX (epithelial restricted with serine box) oncoprotein which has been reported to be stimulated in the early stages of breast cancer and to be activated by the HER2/*neu* breast cancer oncogene [21]. Also the identification of PBEF as an overexpressed gene in CRC is a novel finding. PBEF has been reported as cytokine which is believed to act on early B-lineage precursor cells [22] and is normally expressed in bone marrow stromal cells or activated human lymphocytes. The same sequence has also been reported as a putative lymphocyte Go/G1 switch gene (i.e. G0S9) [23]. Finally, there is an increasing body of evidence relating defects in Smad genes to carcinogenesis and as such, the recovery of Smad5 as a differentially expressed gene product is an important finding. The Smad5 gene belongs to a family of genes that transduce signals from the TGF β family of cytokines. Defects in this pathway are thought to result in non-responsiveness to TGF β. The 612 bp *Rsa*I fragment of the Smad5 gene that we isolated (Col-p19) did not show any mutations as has been reported for Smad2 and Smad4 [24,25]. A recent immunohistochemical study has reported that receptor-activated Smad genes, like Smad5, show increased expression in a fraction of tumor cells compared to epithelial mucosa of normal colon [20].

Cell surface or secreted molecules are ideal targets for immunotherapy. One such target is SPARC, which is a 43 kDa glycoprotein that interacts with the extracellular matrix and is believed to co-ordinate both endothelial cell proliferation and migration in processes such as wound healing and angiogenesis. The level of expression of SPARC has previously been shown to correlate with the progression of colon cancer [15]. Two of the overexpressed genes we have isolated belong to the heat shock family of proteins, Hsc70 and a Hsp90 homologue (Col-p27)(Table 2). Heat shock proteins have been shown to have differential expression in gastrointestinal tumors [26] and there is some evidence that members of this family of proteins are present on the surface of tumor cells [27,28].

In addition to the known genes, we have a substantial list of genes which have as yet no functional annotation and in the majority of cases only have homology to EST sequences in the public databases. We intend to validate these targets by immunohistochemistry using the rapid generation of antibody probes to EST-derived peptides using phage display technology [29].

Due to the heterogeneity of tumors, it would be interesting to produce a SSH-cDNA repertoire from pooled tumor and pooled normal samples which will allow for biasing generically overexpressed cancer genes. However, our data suggest that sampling of only one tumor sample yields exclusively 'generic' markers. This in part could be due to the reverse Northern screening method in that the sensitivity threshold identified only the genes with the strongest differential signal. The rarest transcripts, which may be related to tumor heterogeneity, are not detected. In our analysis, the rare transcripts are probably represented by the 292 clones screened that did not give a hybridization signal. Furthermore, we can extrapolate that at least 41% of these will be differentially expressed but currently lying outside the sensitivity limit of the reverse Northern analysis.

To increase the throughput capacity of SSH, it is possible to combine this technology with microarray analysis. A potential advantage of such an approach is that redundant spotting of highly expressed non-differentially expressed genes is avoided allowing for smaller DNA chips to be produced for high throughput analysis. A recent publication by Yang et al. (1999) [8] has indeed reported the marriage of SSH and cDNA microarray technology for the rapid identification of differentially expressed genes.

We show that SSH in conjunction with an efficient screening procedure is an extremely valuable method to produce a broad profile of cloned differentially expressed genes. Through application of this technique for the first time to primary CRC tumor tissue, we report the isolation of several novel genes of which the differential expression is likely to be physiologically relevant.

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