Expression profile of significant immortalization genes in colon cancer

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Abstract. Cancer is a disease of genomic instability, a multistep process involving numerous mutations and chromosomal aberrations. Telomeres are highly specialized structures at the ends of chromosomes and function to stabilize and protect the ends of linear chromosomes, therefore determining cellular immortalization. Homeostasis of telomere length is a multifactor-dependent process. Since cellular immortalization is an early and essential step towards cancer, the aim of the present study was to determine immortalization genes that are significant in colon cancer and assess their usefulness in the early diagnosis of this tumor. Expression profiles of 119 transcripts known to be involved in cellular immortalization were assessed with oligonucleotide microarrays in 13 probes of colon adenocarcinoma (low and high clinical stages) and 9 probes of controls (normal colon tissue) and were compared among these groups with the use of the Significant Analysis Microarray (SAM) software and independently verified with the effect size parameter. Eighteen genes with significantly differential expression between high clinical stage colon cancer and the control group, and 21 with differential expression between low clinical stage colon cancer and the control group were identified. Nine genes showing altered expression in both low and high clinical stage colon cancer: *ACD* (TPP1), *DKC1* and *ERCC1*, *MYC*, *MAX*, *NBN*, *NOLA2*, *PRKDC* and *HSP82* should, in particular, be the subjects of further studies including QRT-PCR methods.

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Introduction

Cancer is a disease of genomic instability, a multistep process involving numerous mutations and/or chromosomal aberrations (1). Chromosome instability (CIN), resulting in losses or gains of whole chromosomes or their fragments, has been observed in the majority of cancers, at the carcinoma *in situ* stage of carcinogenesis or even earlier (2-4). One of the neoplasms known to be CIN-based is colon cancer (5).

Most genetic aberrations, characteristic of cancers, including aneuploidy, gene amplification, loss of heterozygosity and gene loss, can be initiated by telomere dysfunction (5,6). On the other hand, stability of the telomere length determines cellular immortalization, an early and esential step towards cancer (7).

Telomeres are made of tandem 5'-TTAGGG-3' repeats and a number of associated proteins responsible for their length and structure. By forming a loop structure, the very end of a telomere is protected from a DNA break, thus preventing chromosomes from end-to-end fusions, misrepair and degradation (6). This structure, called telosome (shelterin), is associated with six major proteins: TRF1, TRF2, RAP1, TIN2, POT1 and TPP1, and interacts with other proteins and DNA repair factors.

In immortal cells, loss of telomeric DNA due to degradation or incomplete replication is balanced by telomere elongation by DNA polymerase called telomerase (8), which is composed of the telomerase RNA (hTR) and the catalytic subunit, telomerase reverse transcriptase (hTERT) (9,10). It is believed that telomerase is stringently repressed in normal human somatic tissues, but is reactivated in approximately 85-90% of human cancer tissues (11,12). Reported data indicate the strong correlation between telomerase expression, telomere length maintenance and carcinogenesis (13,14). Approximately 70% of immortalized human cell lines and 85-95% of human cancers exhibit a high expression level of hTERT (15). Yet, hTERT or telomerase appears to be insufficiently sensitive to be specific markers of carcinogenesis, particularly in telomerase-negative tumors where alternative lengthening of telomeres (ALT) occurs (16,17). Indeed, telomere length homeostasis is complex and involves

Table I. Factors involved in hTERT and hTR expression, main telomerase subunits.

Gene symbol	Factor name	Reference	Role in transcription	Function in promoter region
CDKN1A	p21	(38)	Activator/inhibitor	Indirectly, cell cycle regulator, by MAP kinase
CDKN2A	p16	(39-41)	Activator/inhibitor	Indirectly, cell cycle regulator, by MAP kinase
E2F1	E2F1	(42,43)	Activator/inhibitor	Directly, transcription factor, inhibits hTERT transcription in cancer, activates it in normal cells
EGFR	EGFR	(44,45)	Activator	Indirectly, by activation of ETS
ESR1	ER	(46,47)	Activator	Directly
ETS1	ETS	(48,49)	Activator	Directly
HDAC9	HDAC	(50-52)	Activator	Indirectly, epigenetic regulation
HIF1A	HIF1	(53)	Activator	Directly, mainly hTR regulation
IGF1R	IGF1R	(45,54,55)	Activator	Indirectly, by IGF1 and MAP kinases
MAD1L1	MAD1	(56)	Activator	Directly, complex with MAX, region E-box
MAP3K	MAP3K	(44)	Activator/inhibitor	Indirectly, by growth factors
MAX	MAX	(57-59)	Activator/inhibitor	Directly, complex with cMYC and MAD1, region E-box
MYC	cMYC	(57-59)	Activator	Directly, complex with MAX, region E-box
MZF		(49)	Inhibitor	Directly
NFKB1	NFKB	(60)	-	Directly
RB1	RB1		Inhibitor	Indirectly, cell cycle regulator, by E2F1 factor
SP1	SP1	(58,59)	Activator	Directly, transcription factor, region GC-box
SP3	SP3	(59,61)	Inhibitor	Directly, transcription factor, region GC-box
TGFBR2	TGFßR	(45)	Inhibitor	Indirectly, by TGFß, cMYC
TP53	TP53	(62)	Inhibitor	Indirectly, cell cycle regulator, by cMYC factor
USF1	USF1	(63,64)	Activator	Directly
USF2	USF2	(63,64)	Activator	Directly, transcription factor
WT1	WT1	(65)	Inhibitor	Directly, transcription factor

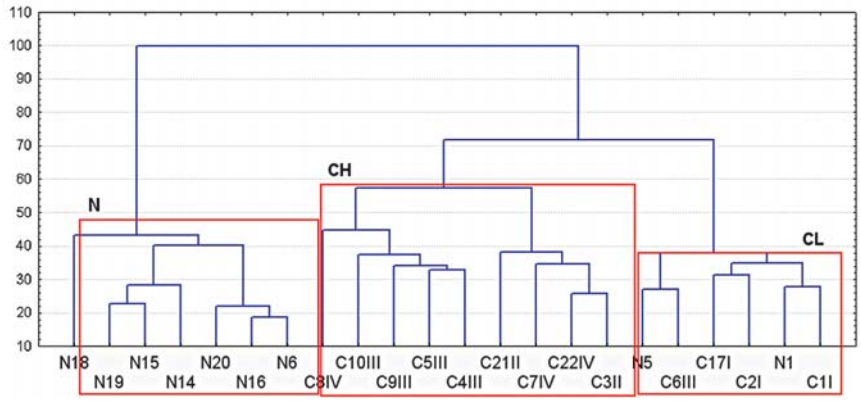

Figure 1. A dendrogram featuring clusters of transcriptoms with regard to clinical diagnosis; Ward's method. C, colon cancer; CH, high stage colon cancer group; CL, low stage colon cancer group; N, control group. Arabic numbers, patient numbers; Roman numerals, colon cancer clinical stage.

hTR and hTERT gene amplification, regulation of their transcription (directly by transcription factors and indirectly by hormones and growth factors), epigenetic modulation, alternative splicing of hTERT, other posttranscriptional modulations of main telomerase subunits and components, regulation of telomerase complex activity, translocation of the telomerase complex, interaction of cell cycle regulators and telosome proteins (10,18-21).

Table II. Growth factors involved in telomerase expression and regulation of its activity.[a]

Gene symbol	Factor name	Effect of action	Mediator	Reference
TGFB1	TGFß	Decreases telomerase activity	c-Myc	(66)
EGF	EGF	Increases telomerase activity in telomerase-positive cells	EGFR, Ras/MEK/ERK, Ets	(44,45,48)
IGF1	IGF1	Increases telomerase activity in prostate cancer cells	Androgen receptor Pi-3-Akt kinase cascade	(45,54, 55)
IGFBP-2	IGFBP-2	Decreases telomerase activity in normal cells		(45,54,55)

[a]According to ref. 45 with modifications.

As cellular immortalization plays an important role in chromosome instability and carcinogenesis, knowledge of the mechanisms of telomere dysfunction seems crucial to understand neoplasm formation and to discover early clinical markers of cancer. The aim of the present study was to identify genes whose expression is significant in colon cancer among 70 candidate genes known to be involved in telomere homeostasis. Fig. 1 shows schematically the complex regulation of telomere stability indicating distribution, role and function of the genes analyzed in our study.

Materials and methods

Thirteen homogenates of G1-G2 colon adenocarcinoma tissue, and 9 of normal colon mucosa were obtained from patients >50 years of age and were histopathologically verified. Colon cancer samples were staged according to the 0-IV clinical staging scale.

Microarray methods were applied with the use of HG_U133A microchips (Affymetrix) according to the manufacturer's protocol. Normalization of microarray results was performed with the use of MicroArray Suite 5.0, R with Bioconductor modules and RMAExpress software.

The studied transcriptomes were clustered using hierarchical agglomeration (Ward's method). As shown in Fig. 1, three clusters of genetic similarity were identified. This allocation was largely compatible with histopathological diagnosis, and the dissected groups of transcriptomes were classified as high stage colon cancer (CH; stages II-IV), low stage colon cancer (CL; stage I and two controls), and normal colon mucosa (N; remaining controls). One stage III colon cancer transcriptom that was clustered with low stage colon cancers was classified as an outlier and excluded from further analysis.

Expression profiles of 119 transcripts (within HG_U133A microchip transcripts), known to be involved in cell immortalization processes (70 genes), were compared among the identified groups with use of the Significant Analysis Microarray (SAM) software. The 'score' and q-value parameters were estimated and verified with ES (effect size) and p-value parameters, independently.

The analyzed transcripts included factors involved in hTERT and hTR expression (Table I), growth factors engaged in telomerase expression and regulation of its activity (Table II), the telomerase complex elements and modulators (Table III), the telosome proteins (Table IV), and proteins associated with telomeres that are involved in DNA repair pathways (Table V).

Table III. Telomerase complex factors and modulators.

Gene symbol	Factor name	Reference
Main telomerase subunits		
TERC	hTR	(67)
TERT	hTERT	(69)
hTR subunit stabilization proteins		
NOLA1	NOLA1	(10,70)
NOLA2	NOLA2	(10,70)
NOLA3	NOLA3	(10,70)
DKC1	DCK1	(28,67)
RPL22	L22	(74-76)
STAU1	STAU	(74,75)
Heterogeneous nuclear ribonucleoproteins involved in telomerase activity regulation		
HNRPA1	hnRPA1	(72)
HNRPC	hnRPC	(72,78)
HNRPD	hnRNPD	(72,80)
Other hTERT complex proteins		
HSP82	HSP90	(67,68)
HSPA9	HSPA9B	(67)
LOC375931	p23	
TEP1	TEP1	(67)
SMG6	hEST1A, C17orf31	(71,72)
SMN1	SMN1	(73)
Phosphorylation factors of telomerase complex		
AKT1	AKT	(77)
PRKCB1	PKC	(77,79)
PPP2R2B	PP2A	(81)
ABL1	c-Abl	(82)

Table IV. Telosome proteins.[a]

Gene symbol	Factor name	Reference	Interactions with other telosome proteins	Proposed mechanism of action	Telomere dysfunction when altered
TERF1	TRF1	(25,83-86)	Stable part of telosome, adheres to dsDNA, interaction with TRF2, multiple interaction with other telosome proteins	Telomere length regulation, 3D structure of telomere stabilization, prevent telomerase access to telomere	Shortening - lengthening
TERF2	TRF2	(25,86-88)	Stable part of telosome, adheres to dsDNA, functional interaction with TRF1, multiple interaction with other telosome proteins mainly with DNA repair system proteins	3D structure of telomere stabilization, prevent telomerase access to telomere	Shortening - lengthening
TERF2IP	RAP1	(72,89)	Stable part of telosome, interaction with TRF2	Negative regulator of telomerase	Lengthening
TNKS	TANK1	(86,90-92)	Unstable part of telomere	Inhibition of telomere - TRF1 interaction	Shortening
TNKS2	TANK2	(91-94)	Unstable part of telomere	Inhibition of telomere - TRF1 interaction	-
RIF1	RIF1	(72,95)	Unstable part of telomere, interaction with RAP1	Telomere length regulation	Lengthening
POT1	POT1	(96,97)	Stable part of telosome, adheres to ssDNA, interaction with TRF1 through TPP	Stabilization/protection 3" overhang, 3D structure of telomere stabilization, prevent telomerase access to telomere, regulation of telomerase activity, TRF1 complex	Shortening - lengthening
PINX1	PINX1	(98)	Stable part of telosome, interaction with TRF1, TRF2, TPP1	Stabilization of telomere by TRF1/2 interaction through TIN2, direct telomerase inhibitor	Lengthening
TINF2	TIN2	(99,100)	Stable part of telosome, interaction with TRF1, TRF2	Stabilization of telomere by TRF1/2, TRF1 activation	Lengthening
ACD	TPP1, PTOP, TINT1	(19,29,101-103)	Stable part of telosome, interaction with TRF1, POT1, TIN2	Stabilization of telosome, 3D structure of telomere stabilization, regulation of telomerase activity, takes part in POT1 translocation toward nucleus	Shortening - lengthening

[a]According to ref. 34 with modifications.

Results

Eighteen genes with significantly differential expression between high clinical stage colon cancer and the control group, and 21 with differential expression between low clinical stage colon cancer and controls were identified among 119 transcripts involved in immortalization processes. Table VI shows the results of the comparative analysis among the groups, with significance criteria: SAM q-value <5%, SAM score >1.5, ES >1.0 and $p<0.05$ (22,23).

Table V. Proteins associated with telomeres that are involved in DNA repair pathways.[a]

Gene symbol	Factor name	Reference	DNA damage function	Interaction with telosome	Telomere dysfunction when altered
ATM	ATM	(72)	Central DNA damage protein	TRF2	Shortening
BRCA1	BRCA1	(72)	HR	TRF2	Lengthening
ERCC1	ERCC1/XPF	(104)	NER	Multiple interaction	Formation of TDM
MRE11A	MRN complex (MRE11)	(36,105)	DSB	Multiple interaction	Shortening - lengthening
NBN	MRN complex (NBS1)	(36,106)	DSB	Multiple interaction	Shortening - lengthening
PARP1	PARP1	(72)	BER	TRF2	None
PARP2	PARP2	(72)	BER	TRF2	None
PRKDC	DNA-PKcs	(72)	NHER	Multiple interaction	Lengthening
RAD50	MRN complex (RAD50)	(105)	DSB	Multiple interaction	Shortening - lengthening
RAD51L3	RAD51D	(107)	HR	Multiple interaction	Shortening - lengthening
RAD54L	RAD54	(108,109)	DSB	Multiple interaction	Shortening - lengthening
WRN	WRN	(110,111)	Helicase	TRF2	Shortening
XRCC5	KU80	(112)	NHER	Multiple interaction	Shortening - lengthening
XRCC6	KU70	(112)	NHER	Multiple interaction	Shortening - lengthening

[a]According to ref. 91 with modifications. DSB, double strand breaks; NER, nucleotide excision repair; NHER, non-homologous excision repair; BER, base excision repair; HR, homologous repair; TDM, telomeric DNA-containing double minute chromosomes.

Discussion

Contribution of telomeres to genomic instability and their role in cellular immortalization processes are possible mechanisms leading to carcinogenesis. Telomere shortening or their dysfunction may result in cancer. On the other hand, telomere length maintenance is regarded as a necessary element of oncogenesis (6,7). The analysis of the expression profile of genes involved in telomere homeostasis may help understand this paradox and discover new tools for the diagnosis and therapy of colon cancer, a model of carcinogenesis in this study.

In the present study, we demonstrated (in a semi-quantitative screening method of oligonucleotide microarrays) that some of the examined transcripts involved in immortalization processes are indicative of colon cancer and are therefore candidates for markers of high or low stage colon cancer. Although the results warrant confirmation in larger study groups, they provide important knowledge of the general trends in expression profiles of immortalization factors. Our broad investigation of cellular immortalization, not only limited to main telomerase subunits but extended to other known telomere homeostasis factors, resulted in valuable findings. Figs. 2 and 3 show schematically genes whose expression was significant under the analyzed clinical conditions.

Telomerase activity and the hTERT expression level have been considered sensitive markers of cancer (yet are not specific to the type of neoplasm) (11,12,24,25). Notably, in our study, hTERT expression was not a significant discriminator between the studied groups, although it has to be mentioned that the second main telomerase subunit, hTR, was not present on the microchip. Instead various other factors composing the telomerase complex appeared important in colon cancer; such as Dyscerin (DKC1), which was a strongly significant positive differentiator for both CL and CH groups vs. the control. Cohen *et al* (26) found that DKC1 was one of three (beside telomerase reverse transcriptase and telomerase RNA) critical components of the telomerase complex. In light of this finding, DKC1 appears to be among the most important candidates warranting further investigation. Mutations in DKC1 have been found in the rare disease, dyskeratosis congenita (27), which is also related to telomerase and telomere homeostasis dysfunction (28).

Another component of the hTR complex is NOLA2, a member of the same group of proteins as DKC1 and snoRNPs (10). Its expression was found to be significant in high stage colon cancer. It may therefore be useful in the assessment of disease progression. Other factors significant in the CH group were also found, such as the Hsp90 (*HSP82*) protein in the hTERT complex. However, strict involvement of DKC1 and NOLAs in telomerase activation (10) makes them potential markers with greater specificity.

An additional important factor, specific to telomere length maintenance, is the telosome component TPP1 (*ACD1*) which was a positive significant gene in both colon cancer groups in our study. TPP1 is considered a key mediator between main telosome components TRF1, TRF2 and POT1 (19,29), and also, by interaction with TIN2, it stabilizes a high level conformation of telosome (30). Its increased expression

Table VI. Transcripts of significantly different expression between low stage colon cancer and control groups (CL vs. N), and between high stage colon cancer and control groups (CH vs. N), according to 'score' parameter (SAM analysis) for q-value <0.05.[a]

Gene name	Group	SAM score	
		CL vs. N	CH vs. N
ACD	T	1.460	3.240
AKT1	PH	-2.170	
CDKN1A	CC	-2.240	-3.410
CDKN2A	CC		1.800
DKC1	RS	3.240	3.040
EGFR	GF	-2.190	-3.440
ERCC1	T		2.320
HIF1A	TF	2.020	
HNRPC	RS	2.260	
HNRPD	RS	2.600	
HSP82	CS	2.540	
HSPA9	CS	2.140	
IGF1R	GF		-1.870
IGFBP2	GF	2.900	
MAX	TF	-2.690	-3.660
MRE11A	T		2.220
MYC	TF	3.037	4.780
NBN	T	1.910	1.610
NOLA1	RS	2.070	
NOLA2	RS	2.710	
PARP1	T	1.900	
PRKDC	T	1.950	
RAD50	T		1.850
RAD51L3	T	1.570	1.980
RAD54L	T	2.400	2.950
SP1	TF		-2.520
STAU1	RS		1.770
TGFB1	GF		1.840
WT1	TF		1.960
XRCC6	T	1.750	

[a]'Score' indicates a rate of significance, positive values indicate overexpression of transcripts, and negative values, silencing of transcription. Groups of factors: CC, cell cycle factors; CS, catalytic hTERT subunit of telomerase; GF, growth factors; PH, phosphorylation factors; RS, RNA subunit of telomerase; T, telosome; TF, transcription factors.

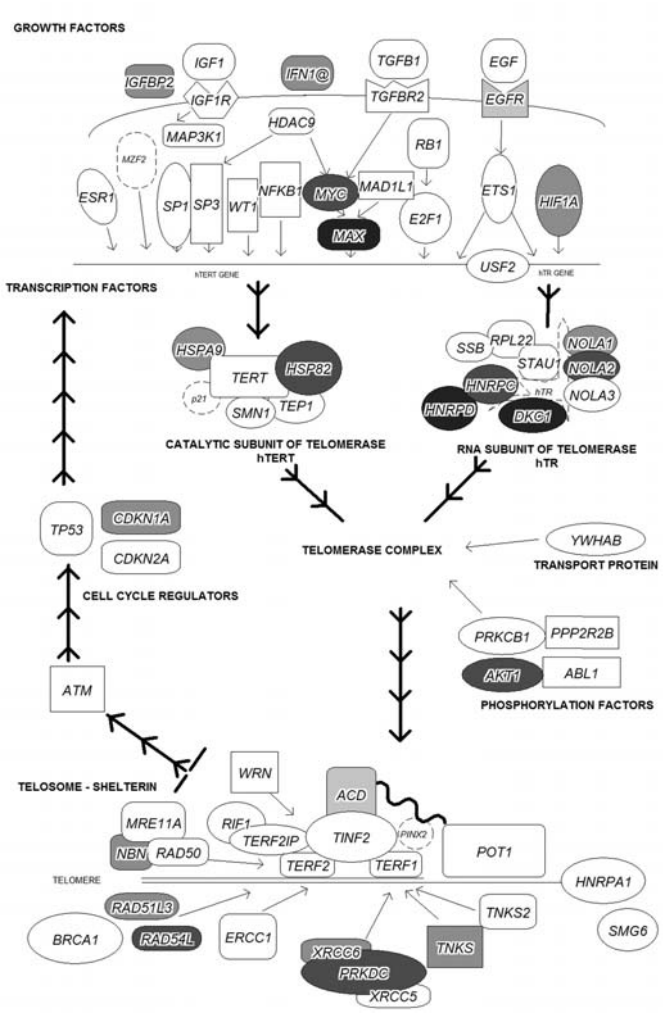

Figure 2. Transcripts differing significantly between the low stage colon cancer and control groups according to 'score' parameter (SAM analysis) for a q-value <0.05. Intensity of grey indicates the degree of significance. Shape of figures: oval, telomerase/telomere lengthening activator; rectangle, telomerase/telomere lengthening inhibitor; other shapes, multiple actions; dashed outline, factor not present on microchip.

in cancer may reflect telomere disturbances resulting from chromosomal instability and makes it an attractive candidate marker of carcinogenesis.

Notable results have been obtained with some pleiotropic factors, such as cofactors c-Myc/Max, which are transcription factors of hTERT and hTR (31,32). Myc was a positive significant gene in the studied colon cancer groups. It has been postulated that c-Myc acts as a transcription factor of many genes, but its contribution to oncogenesis is most likely through telomerase promoter activation (31,33). Some authors have shown a correlation between hTERT and c-Myc overexpression in prostate (34) and cervical cancer (35). In our study, not only c-Myc overexpresssion did not correlate with higher hTERT expression level, but Max protein appeared a strong negative significant factor in both CH and CL groups. Is it simply a negative feedback, or rather a hallmark of more sophisticated mechanisms of Myc/Max complex regulation? It has also been suggested that the transforming activities of Myc extend beyond its ability to activate hTERT gene expression and hence telomerase activity (32). However, such strong associations with colon cancer indicate importance of these transcription factors for further investigation. The other pleiotropic factors contributing to immortalization in our study were significant DNA repair family proteins *ERCC1*, *PRKDC*, *RAD51L3*, *RAD54L* and *NBN*. All of them interact with stable proteins of telosome. Particularly, nibrin (NBS1, *NBN*) warrants further analyses, due to its influence on alternate lengthening of telomeres

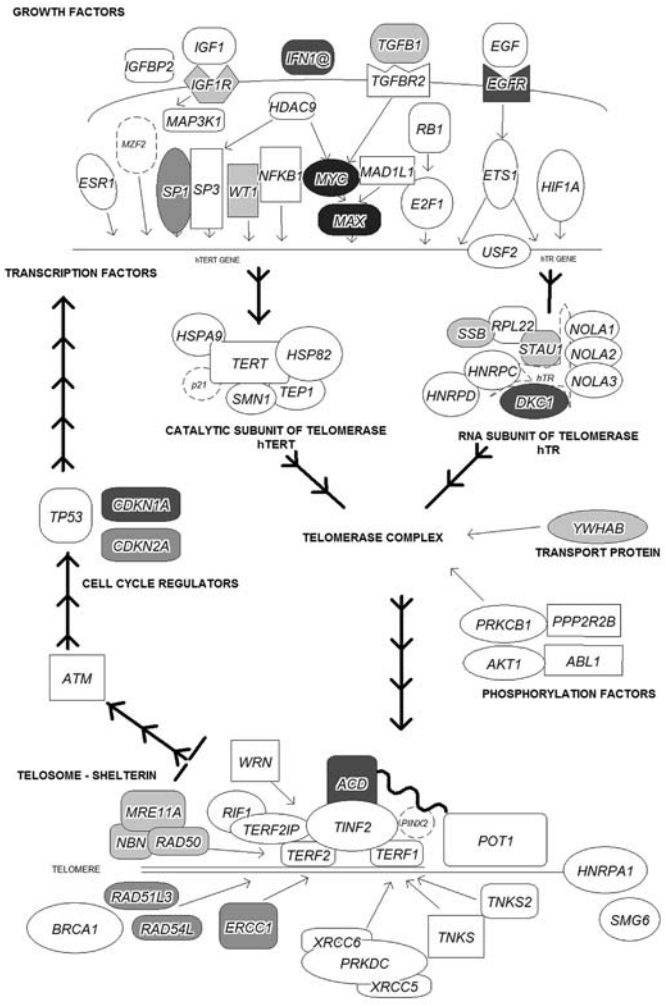

Figure 3. Transcripts differing significantly between the high stage colon cancer and control groups according to 'score' parameter (SAM analysis) for a q-value <0.05. Intensity of grey indicates the degree of significance. Shape of figures: oval, telomerase/telomere lengthening activator; rectangle, telomerase/telomere lengthening inhibitor; other shapes, multiple actions; dashed outline, factor not present on microchip.

(ALT) (36). ALT is independent of telomerase activation, and it is noted in approximately 10% of neoplasms (16,17). The most popular hypothesis of the mechanism of ALT is homologous recombination between ends of chromosomes (37), which NBS1 is postulated to take part in. In the context of our results, NBS1 appears a significant factor with the potential for discerning cases of cancer that are based on ALT mechanisms of immortalization.

Division of the study group according to transcriptive similarity, albeit highly compatible with clinical division, imposed inclusion of two control cases in the low stage colon cancer group, which can be considered a limitation of this study. However, as these two transcriptomes came from samples of normal colon mucosa of patients with colon cancer (Fig. 1), we assumed that these two transcriptomes featured a transcriptive profile of early oncogenesis prior to morphological manifestation.

The mechanisms that induce and influence genomic instability and immortalization processes in cancer, in general and more specifically in colon cancer, are only partly understood. Although our data are preliminary, we believe that the knowledge gained from our study may aid in the diagnosis of cancer and the development of therapeutic tools, as well as provide a novel insight into cell immortalization processes.

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