Regulation of protein tyrosine kinases in tumour cells by the transcription factor Ets-1

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Abstract. Tyrosine phosphorylation is one of the key covalent modifications that occurs in multicellular organisms as a result of intercellular communication. The family of tyrosine kinases (PTKs) are responsible for part of the cellular phosphorylation and are involved in a broad variety of cellular functions including differentiation, proliferation, migration, invasion, angiogenesis and survival under physiological as well as pathological conditions. Aberration in PTK signalling occurs in inflammatory diseases and diabetes, and aberrant expression can lead to benign proliferative conditions as well as to various forms of cancer. Indeed, more than 70% of the known oncogenes and proto-oncogenes involved in cancer code for PTKs. Therefore, these enzymes are now used as targets in the treatment of different tumours. Ets-1 is a transcription factor expressed in a number of human malignancies with demonstrated roles within both neoplastic cells and tumour stroma. These roles include stimulation of tumour cell proliferation and invasion as well as tumour angiogenesis. Database searches have revealed that ETS binding sites are present in several promoters of PTK-encoding genes. We investigated the role of Ets-1 in transcriptional regulation of a panel of 89 PTKs in epithelial HeLa tumour cells. In this study, HeLa cells stably overexpressing and underexpressing Ets-1 were used for real-time PCR analysis of all known human PTKs. The results suggest that Ets-1 is an essential transcription factor that cannot be substituted by other members of the ETS family. Transcription of most PTKs was found to be increased by Ets-1. In contrast Ets-1 seems to act as a transcriptional repressor of other PTKs. The data presented here underscore the importance of Ets-1 in tumour development and progression.

Introduction

Protein tyrosine kinases (PTKs) are enzymes that catalyze the transfer of the γ phosphate of ATP to tyrosine residues on protein substrates. By phosphorylation of tyrosine residues on receptors or downstream signalling proteins, their enzymatic activity is modulated (1,2). PTKs are involved in most cellular signalling pathways and regulate many key functions such as proliferation, differentiation, migration, metabolic changes and apoptosis (3-14). New blood vessel development relies on the concerted action of several subfamilies of PTKs and their cognate ligands as well (15). Due to the involvement of PTKs in such important processes, a stringent regulation of their expression and activity is necessary for maintaining normal cellular functions (16). The importance of PTKs in health and disease is further underscored by the existence of aberrations in PTK signalling occurring in cancer, inflammatory diseases and diabetes (17-19). Dysregulation of PTKs through point mutations or overexpression can lead to various forms of cancer as well as benign proliferative conditions (5,20-22). Indeed, more than 70% of known oncogenes and proto-oncogenes involved in cancer encode PTKs (23). Another oncogenic mechanism involving PTK are translocations creating fusion proteins with constitutive expression or activity. Among such translocations are ETV-6/ABL1, ETV-6/FRK, TEL/PDGFR-ß fusing translocated PTKs and members of the ETS transcription factor family (24-28). ETS family members are widely expressed and are involved in basic cellular functions such as proliferation, apoptosis, angiogenesis, differentiation and migration (29-40). These functions are critical for the development of cancer (41). All ETS members share a specific DNA binding domain, called the ETS domain, which consists of approximately 80 amino acids with 4 tryptophane repeats (42). It has been shown that two-thirds of the 27 human *ets* genes are expressed ubiquitously and that the DNA binding of ETS proteins has overlapping specificities (43).

Several PTKs interact with the Ras/Raf/MAP or with the MEK1/ERK1/2 pathway which is known to result in transcriptional activation of Ets-1 (44-50) which is the prototype of the ETS transcription factor family (51).

Furthermore, ETS binding sites have been found in several PTK promoters (52-58) suggesting a role for Ets-1 in the transcriptional regulation of PTKs. Since an expression analysis of all known PTKs in correlation to the expression level of Ets-1 in human tumour cells has not yet been carried out, we determined expression levels of all known PTKs by real-time PCR in stably Ets-1-overexpressing and -underexpressing HeLa cells which we have used and characterized in previous studies (32). We showed that Ets-1 is an essential

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transcriptional activator for most PTKs including EGFR, FGFR4, JAK3, c-KIT and MET, while the expression of 10 out of 89 PTKs (including EPHA3, EPHA4, EPHB2, EPHB3 and PDGFR-ß) were suppressed. These data suggest a regulatory role of Ets-1 in a number of PTKs involved in tumour development and progression.

Materials and methods

Cell culture. The establishment of Ets-1-overexpressing and -underexpressing HeLa cell lines has been previously described (32). Briefly, the plasmids pcDNA3.1*h-ets-1* and pcDNA3.1*h-ets-1* inverse, respectively, were transfected into the cells by the calcium phosphate method (59). Twenty-four hours after transfection, selection was started using 400 μg/ml G418 (Invitrogen). Ets-1-overexpressing (HeLa Ets-1) and -underexpressing (HeLa inverse) cells were selected from pooled populations of transfected cells in order to avoid clonal variations.

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with antibiotics and 10% heat-inactivated fetal calf serum.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from cell monolayers (RNeasy Kit, Qiagen), and the quality was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies).

For samples with a RIN factor >9, cDNAs were constructed by reverse transcription in a 10-μl reaction volume containing 2 μg of total cellular RNA, 1 μl dNTPs (10 mM), 1 μl of random hexamer primers (10 mM), and RNase-free water. After incubation at 65˚C for 5 min, the reaction mixture was placed on ice for 1 min. Then 2 μl of RT buffer (Invitrogen), 4 μl $MgCl_2$ (25 mM), 2 μl of 0.1 mM DTT and 1 μl RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) were added. After incubation at 42˚C for 2 min, 1 μl of Superscript II Reverse Transcriptase (50 units/μl) (Invitrogen) was added for the transcription at 42˚C for 1 h. Inactivation of the enzyme was performed by heating at 70˚C for 15 min. RNA was removed by addition of 1 μl RNaseH and incubation at 37˚C for 20 min.

Real-time RT-PCR. PCR reactions were performed using the TaqMan Low Density Array System (Applied Biosystems) (60-63). Arrays with four sets of 96 genes were designed, and each reaction was performed in duplicate. Primer/probe sets were selected from a large pool of TaqMan Gene Expression Assays (Applied Biosystems) that are predesigned and tested to strictly match system-immanent criteria of uniform PCR temperature profile and PCR efficiency. Beside the 89 tyrosine kinases, 5 putative endogenous control genes (HPRT1, UBC, G6PDH, RNA-polymerase II, 18sRNA) were included on each array. All amplicons span an exon-exon-junction to achieve mRNA specificity and are of two-digit-bp length. Primer/probe sets are spotted on a custom 384-well card during fabrication at the manufacturer. cDNA was diluted to a final concentration of 8 ng/μl and mixed 1:1 with 2X TaqMan Universal PCR Master Mix (Applied Biosystems). One hundred microliters containing 400 ng cDNA was loaded into each fill port and

A

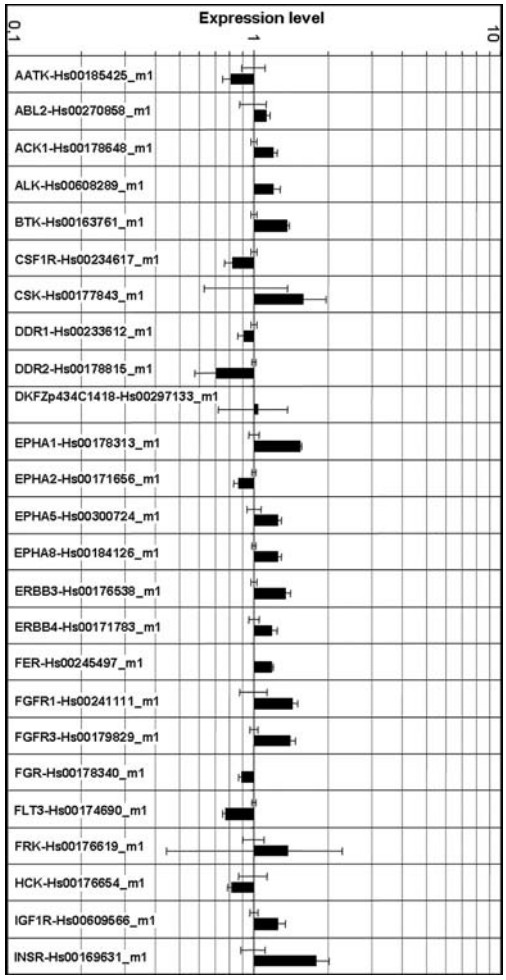

Figure 1A.

distributed through microchannels into the 48 reaction wells per filling port by centrifugation. Subsequent sealing of the microchannels prevented cross-contamination during PCR. The arrays were thermal cycled at 50˚C for 2 min and 94.5˚C for 10 min, followed by 40 cycles at 97˚C for 30 sec and 59.7˚C for 1 min on an Applied Biosystems 7900HT instrument.

Data processing. Absolute Ct-values were calculated using SDS 2.2 Software (Applied Biosystems) and transferred into the qBase Software (Micosoft Excel-Plug-In, Jo Vandesompele) for further analysis. Since normalization based on single housekeeping genes may not provide accurate results, we used a geometric mean of RNA-polymerase II and 18sRNA for normalizing, which showed the most stable expression across all samples. Calculations were based on an amplification efficiency of 1.95 which represents quite exactly the tested amplification efficiency of pre-designed TaqMan-assays (1.9-2.0, according to the manufacturer's instructions). The expression levels of the target genes were given relative to the calibrator sample HeLa Ets-1 with the standard error of mean calculated from replicate accuracy.

Immunocytochemistry. Cells grown in 1-cm^2 culture chambers (Nunc) were rinsed once with phosphate-buffered saline

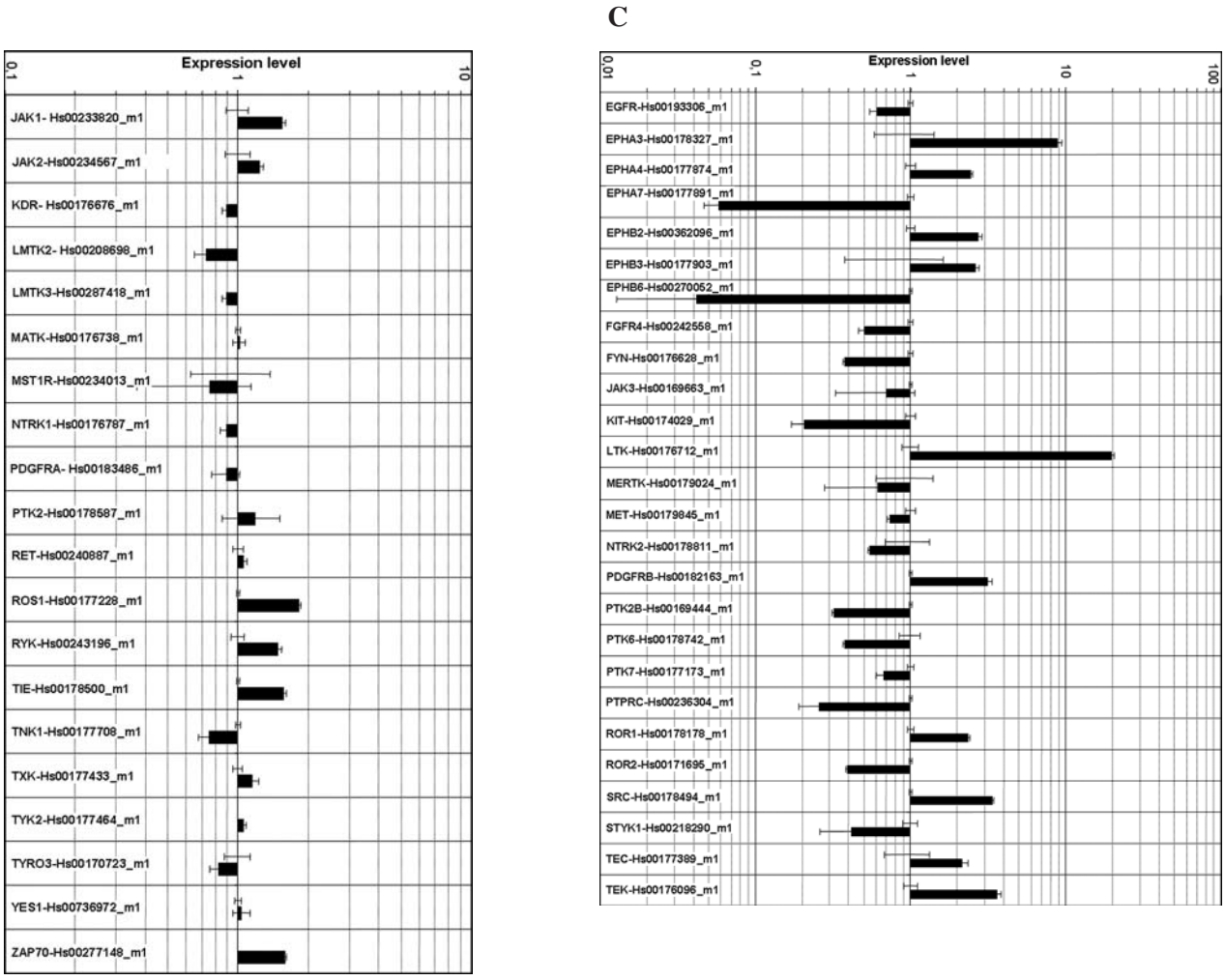

Figure 1. Real-time RT-PCR analysis of human PTKs. RT-PCR was performed with total RNA from HeLa Ets-1 and HeLa inverse cells, respectively, using the TaqMan Low Density Array System. The expression of 89 genes coding for human PTKs was measured in parallel. The mean value of 2 x 2 independent measurements is shown. The expression level in HeLa Ets-1 cells was set to 1. PTK expression levels in HeLa Ets-1 inverse cells are shown in relation to the expression rate in HeLa Ets-1 cells. In A and B, differentially expressed PTKs exhibiting a difference of ≤1.5 and in C, ≥1.5 between HeLa Ets-1 and HeLa Ets-1 inverse cells are shown.

(PBS) (Life Technologies), twice with 70% ethanol and fixed in a mixture of ice cold methanol and ethanol (1:2 v:v) at 4˚C for 20 min. After washing twice with PBS, cells were stained with antibodies directed against PDGFR-ß (1:1000 dilution) (Santa Cruz Biotechnology), EGFR (1:25 dilution) (Dako), ZAP-70 (1:25 dilution) (Biosystems) or c-kit (1:25 dilution) (Dako). The cells were incubated with the primary antibody at 4˚C for 12 h. Detection of the primary antibody was carried out using the LSAB-peroxidase System (Dako) according to the manufacturer's instructions.

Results

We previously demonstrated effective overexpression or blockade of Ets-1 protein in HeLa Ets-1 and HeLa inverse cells (32). When examined by Western blotting, expression levels of proteins with phosphorylated tyrosine residues showed variations between wild-type (HeLa) cells, HeLa cells overexpressing (HeLa Ets-1) and underexpressing Ets-1 (HeLa inverse) (data not shown). We then evaluated the effect of Ets-1 on the mRNA expression of all known human protein tyrosine kinases (PTKs) by comparing HeLa Ets-1 and HeLa inverse cells.

As shown in Fig. 1, a broad variety of PTKs are differentially expressed in Ets-1-overexpressing und -underexpressing HeLa cell lines. The expression level in HeLa Ets-1 cells was set to 1. The PTK expression levels in HeLa inverse cells are shown in relation to the expression rate in HeLa Ets-1 cells. In Fig. 1A and B the differentially expressed PTKs are summarized and show only a small difference (≤1.5) between the two cell lines. In Fig. 1C the PTKs showing a ≥1.5-fold change in expression are listed. It is evident that PTKs are regulated at the mRNA level by Ets-1. Ten PTKs (EPHA3, EPHA4, EPHB2, EPHB3, LTK, PDGFR-ß, ROR1, SRC, TEC and TEK) had a higher RNA expression level in the HeLa inverse compared to the HeLa Ets-1 cells (Fig.1C). This suggests that the transcription of these PTKs is repressed by Ets-1. In contrast, 16 PTKs (EGFR, EPHA7, ERHB6, FGFR4, FYN, JAK3, c-KIT, MERTK, MET, NTRK2, PTK2B, PTK6, PTK7, PTPRC, ROR2 and STYK1) were found to have a reduced RNA expression level in the HeLa inverse compared to the HeLa

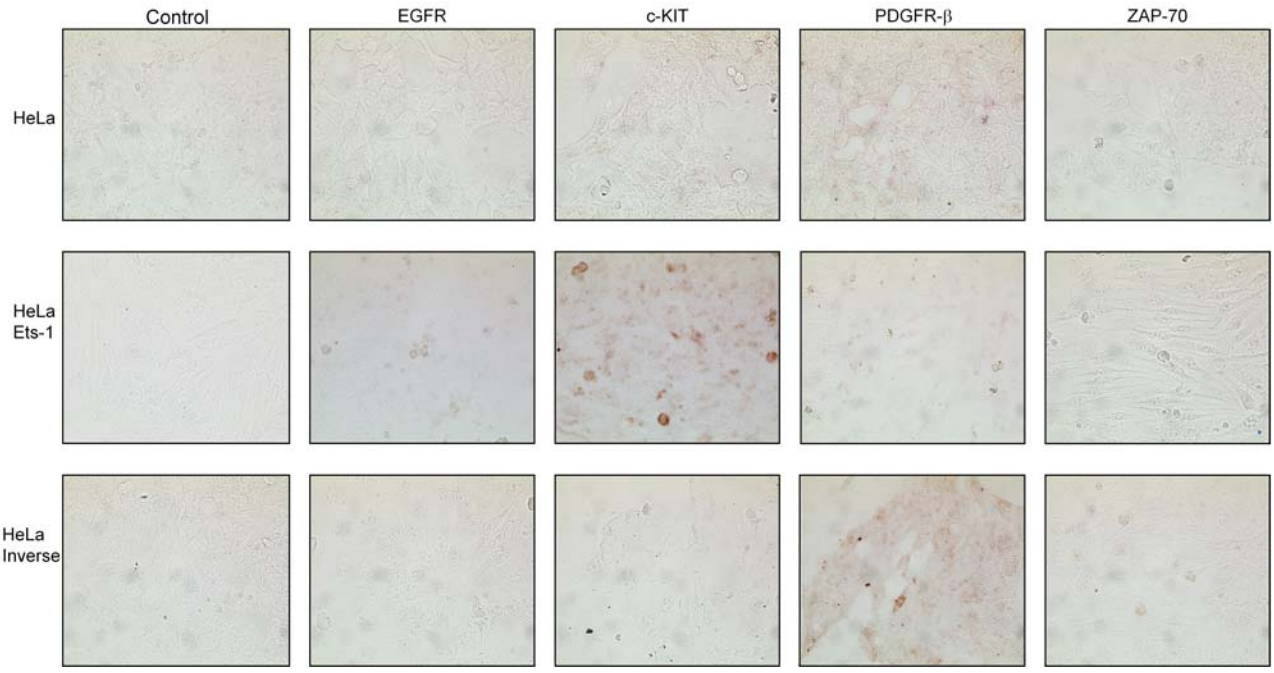

Figure 2. Immunohistochemical staining for EGFR, c-KIT, PDGFR-ß and ZAP-70 expression in HeLa, HeLa Ets-1 and HeLa Ets-1 inverse cell lines. Cells were fixed and stained with antibodies against EGFR, c-KIT, PDGFR-ß and ZAP-70. Images were captured at a 30-fold magnification.

Ets-1 cells (Fig. 1C). For these 16 PTKs, Ets-1 seems to be an essential transcriptional activator.

Twenty PTKs were not expressed in either cell line. These PTKs were ABL1, AXL, BLK, BMX, EPHB1, EPHB4, ERBB2, FES, FGFR2, INSRR, ITK, LCK, LYN, MERTK, MUSK, NTRK3, SRMS, SYK, VEGFR-1 and VEGFR-3.

The differential expression of selected PTKs was verified at the protein level by immunocytochemistry for EGFR, c-KIT, PDGFR-ß and ZAP-70 (Fig. 2). Expression in the parental HeLa, HeLa Ets-1 and HeLa inverse cell lines is shown. Expression of EGFR was hardly detectable in HeLa Ets-1 cells. Wild-type HeLa cells, as well as HeLa inverse cells did not show any expression of EGFR.

In contrast, an increased c-KIT expression was found in HeLa Ets-1 cells compared to HeLa and HeLa inverse cells. PDGFR-ß expression was enhanced in HeLa inverse cells in comparison to the two other cell lines. No significant ZAP-70 expression was detected in the three cell lines correlating in essence to the results at the mRNA level.

In conclusion, differences in expression of 4 PTKs at the RNA level were observed at the protein level by immunocytochemistry.

Discussion

The Ets-1 gene has first been identified as the cellular precursor of the viral Ets-1 oncogene which, together with v-Myb, induces a mixed erythroleukemia in chickens (64). Ets-1 is now considered to be the prototype of the ETS family of transcription factors which now includes approximately 30 members (42,51). All ETS transcription factors share a specific DNA binding domain composed of approximately 80 amino acids (ETS domain) (42) which mediates transactivation or repression of numerous target genes (65-71). In previous studies, we and others first related Ets-1 to new blood vessel formation including tumour vascularization (72-75). Subsequent studies revealed Ets-1 expression likewise within fibroblasts in the stroma of invasive human tumours (76). Stromal fibroblasts contribute to tumour invasion by the secretion of different matrix-degrading proteases. Their genes have been shown to be Ets-1 target genes in both fibroblasts and endothelial cells (77-80) which require invasive properties likewise for early steps of angiogenesis (81,82). More rarely than in tumour stroma, Ets-1 is expressed within neoplastic cells themselves, as we previously revealed in human breast cancers and melanomas as well as in rat C6 glioma and human HeLa cells (32,34,83-85).

In human breast cancer cell lines, Ets-1 expression was proposed to be associated with *in vitro* invasiveness and epithelial-mesenchymal transition, linked to expression of vimentin, uPA, MMP-1 and MMP-3 and to a loss of E-cadherin (83). In normal madin-darby canine kidney (MDCK) cells, Ets-1 was a target of scatter factor/hepatocyte growth factor (SF/HGF) signalling through met receptor tyrosine kinase (46-48,86). In these cells SF/HGF-induced Ets-1 expression was correlated with morphological changes through RAS-RAF-MEK-ERK signalling which activates Ets-1 (46-49). By overexpressing and blocking Ets-1 (through RNAi approaches and decoy oligonucleotides), we demonstrated that Ets-1 has roles in cell proliferation, migration and invasion in melanoma, C6 glioma and HeLa cell lines, and we identified several Ets-1 target genes such as MMP-1, -3, -9 and uPA as well as integrin ß2 and ß3 (32,34,84,85).

It is well established that PTKs are involved in most cellular signalling pathways and that they regulate cellular key functions such as proliferation, differentiation, migration, metabolic changes and anti-apoptotic signalling (3-14). In diabetes, inflammatory diseases and cancer, unregulated

Table I. Promoter regions of tyrosine kinases encoding genes obtained from public available sequences (National Center for Biotechnology Information or Ensembl Genome Browser) were searched for the number of potential Ets-1 binding sites with the PATCH 1.0 public program.[a]

Tyrosine kinase	x-fold mRNA expression differences in HeLa inverse compared to HeLa Ets-1 cells	No.of potential Ets-1 binding sites in the promoter region	Described Ets-1 binding sites in the literature
LTK	19.767	2	
EPHA3	8.817	6	(55)
TEK	3.575	7	
SRC	3.331	3	
PDGFR-ß	3.109	2	
EPHB2	2.726	2	
EPHB3	2.624		
4EPHA4	2.445	0	
ROR1	2.361	2	
TEC	2.124	0	
ROS1	1.818	0	
INSR	1.786	2	(53)
ZAP70	1.587	2	
CSK	1.580	5	
TIE	1.568		
JAK1	1.544	2	
EPHA1	1.526	1	
RYK	1.485	1	
FGFR1	1.436	6	
FGFR3	1.389	1	
FRK	1.363	2	
BTK	1.353	1	(92)
ERBB3	1.335	2	
EPHA8	1.246	2	
JAK2	1.242	4	
EPHA5	1.241	1	
IGF1R	1.241	4	
ACK1	1.200	5	
ALK	1.200	3	
PTK2	1.184	1	
ERBB4	1.169	1	
FER	1.169	1	
TXK	1.145	2	
ABL2	1.116	3	
RET	1.057	3	
TYK2	1.052	2	
DKFZp434C1418	1.042	2	
YES1	1.035	1	
MATK	1.010	6	
DDR1	0.907	4	
FGR	0.891	2	
KDR	0.891	6	(90)
LMTK3	0.891	2	
NTRK1	0.891	1	
PDGFRA	0.891	30	(58)
EPHA2	0.866	2	
TYRO3	0.823	3	
CSF1R	0.820	4	(57)
MCK	0.808		
AATK	0.803	3	
FLT3	0.764	1	
MST1R	0.753	3	
TNK1	0.748	1	
MET	0.740	5	(54)
LMTK2	0.731	6	
DDR2	0.701	2	
JAK3	0.696	13	(91)
PTK7	0.665	2	
MERTK	0.612	7	
EGFR	0.609	3	(52)
NTRK2	0.548	3	
FGFR4	0.500	1	(89)
STYK1	0.416	3	
ROR2	0.390	1	
PTK6	0.379	0	
FYN	0.375	4	
PTK2B	0.320	15	
PTPRC	0.256	2	
KIT	0.208	6	(56)
EPHA7	0.058	1	
EPHB6	0.042	2	

[a]The results are shown in relation to expression differences of tyrosine kinase transcripts between HeLa inverse and HeLa Ets-1 cells.

activation of PTKs is often observed (5,17-22,87). More than 70% of known oncogenes and proto-oncogenes involved in cancer code for PTKs (23). Furthermore, as mentioned previously, several types of cancers are caused by fusion proteins composed of translocated PTKs and members of the ETS transcription factor family (24-27). Since protein tyrosine kinases are the main intracellular transducers of signalling pathways leading to the most basic properties of tumour cells (proliferation, migration and invasion), we used HeLa cells to address the question of whether PTK-encoding genes are among Ets-1 target genes.

Our study specifically addressed Ets-1 effects and ruled out any indirect effect that could be mediated by an endogenous activation of Ets-1. This is of particular interest since Ets-1 can act as both an upstream and downstream effector of signalling pathways. As downstream effector its activity is

directly controlled by specific phosphorylations resulting in the ability to activate or repress specific target genes (51). As upstream effector it is responsible for the spatial and temporal expression of numerous growth factor receptors including PTKs (88).

The data presented here show that Ets-1 is involved in the transcriptional regulation of a broad variety of PTKs in HeLa cells. It seems that Ets-1 is an essential transcriptional regulator for most PTKs in these cells and that this role cannot be substituted by other members of the ETS transcription factor family. One or more ETS binding sites have been identified in the promoter regions of several PTKs including EGFR, FGFR4, INSR, MET, EPHA3, c-KIT, CSF1R, PDGFR-α, JAK3, BTK, VEGFR-1 and VEGFR-2 (52-58,72,89-92). The promoter regions of the other PTKs (obtained from public available sequences, National Center for Biotechnology Information or Ensembl Genome Browser) investigated in the present study were analyzed for potential ETS binding sites by the use of the PATCH 1.0 public program. In nearly all PTK promoter regions, potential Ets-1 binding sites were present suggesting direct effects of Ets-1 on the expression levels of PTK-encoding genes. Nevertheless, no direct correlation between the numbers of potential Ets-1 binding sites and the extent of regulation was evident as shown in Table I. For 6 PTK-encoding genes (EPHA4, TEC, ROS1, TIE, MCK and PTK6), no Ets-1 binding sites were detected in the promoter regions. In these cases the effects of Ets-1 on PTK expression was probably indirect and may have been caused by the regulation of other transcription factors and/or essential cofactors (93).

According to the present data, Ets-1 may act either as an activator or repressor for transcription of different PTKs. Such dual effects, transactivation or repression of different target genes by one transcription factor, have previously been described for other transcription factors likewise among which are members of the ETS family (65-71,94).

PTK mRNA expression differences were verified by immunocytochemistry using 4 well-established antibodies (Fig. 2). The mRNA differences were verified for EGFR, c-KIT and PDGFR-ß at the protein level. For ZAP-70 no significant expression was detected in all cell lines correlating in essence to the results at the mRNA level.

In general, gene expression is the result of a cooperated action of many different factors, including both activators and repressors and their competition for specific DNA sequences in promoter regions. The transcription factors or cofactors determining the effect of Ets-1 as a repressor or activator for particular PTK genes remain to be identified.

In conclusion. Ets-1 seems to be involved, to a great degree, in the regulation of expression of many PTKs in HeLa tumour cells and thereby in many networks of intercellular communication important for cancer. These new findings, therefore, underscore the importance of the Ets-1 transcription factor for tumour development.

Acknowledgements

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