ORIGINAL ARTICLE

STAT3 mRNA and protein expression in colorectal cancer: effects on STAT3-inducible targets linked to cell survival and proliferation

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Aims: To evaluate mRNA and protein expression of signal transducers and activators of transcription (STAT)3 in colorectal carcinomas (CRCs) and to define the association of STAT3 activity with the STAT3-inducible targets cyclin D1, survivin, Bcl-xl and Mcl-1.

Materials and methods: Matching serial sections of normal colonic epithelium and invasive CRCs (n=32) were subjected to quantitative reverse transcriptase polymerase chain reaction specific to STAT3, cyclin D1, survivin, Bcl-xl and Mcl-1, as well as immunohistochemistry. For STAT3 immunohistochemistry, two antibodies, recognising unphosphorylated (UP-) and phosphorylated (tyr705, P-) STAT3 were used. Ki-67 (MIB-1) staining was included as a proliferation marker.

Results: Compared with normal colonic epithelium, UP-STAT3 and P-STAT3 (p=0.023 and 0.006) protein expression and expression of its associated targets cyclin D1, survivin and Bcl-xl were significantly (all p<0.001) increased in carcinoma. In carcinomas, STAT3 (p=0.019) and Bcl-xl (p=0.001) mRNAs were correlated with lymph node status. Moreover, nuclear P-STAT3 protein expression (active state) was associated with the expression of its target genes Bcl-xl (p=0.038) and survivin (p=0.01) as well as with Ki-67 (p=0.017). By contrast, cytoplasmic UP-STAT was significantly linked to Bcl-xl mRNA (p=0.024) and protein (p=0.001) as well as to cytoplasmic survivin protein expression (p=0.019).

Conclusion: Both inactive (UP-STAT3) and active (P-STAT3) STAT3 proteins are markedly increased in invasive CRCs. This is associated with Bcl-xl and survivin induction, increased proliferation and lymph node metastasis. This study therefore provides the basis for further examination of the prognostic or predictive value of these molecular markers in CRC.

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Molecules of the signal transducers and activators of transcription (STAT) family have a major role in cytokine and growth factor signalling in normal tissues.^{1,2} Specific dysregulation of STATs and STAT-associated signalling pathways has been observed in diseased states, such as in chronic inflammatory bowel disease³ and malignant transformation.^{4–6} Moreover, aberrations of STAT3 expression and signalling have been identified in haematopathological malignancies, such as multiple myeloma,⁷ and also in a variety of solid cancers, such as tumours of the breast,^{8,9} ovary,¹⁰ pancreas¹¹ and prostate,¹² and melanoma.¹³ As this activation seems to be tumour-specific, STATs may represent a novel molecular target for therapeutic interventions^{14,15} and, indeed, several strategies for inhibition of STAT signalling have recently been tested.^{16–19}

The recent interest in blocking the STAT-signalling cascade in malignant disorders is fuelled by the diverse roles of STAT molecules with respect to cell proliferation and survival, as well as DNA transcription, all processes that are crucial for progression to malignancy. The complex network of STAT signalling is dependent on a series of tyrosine and serine phosphorylation steps, leading to full activation of STAT molecules that translocate from the cell cytoplasm into the nucleus and bind to DNA promoter sequences of STAT-inducible genes.²⁰ Recent data, however, suggest that unphosphorylated (UP-)STAT3 may also induce changes in gene expression,²¹ but the effect of this pathway in cancer is still unclear. Irrespective of the mechanism of nuclear translocation, the specificity of different STAT family members for specific DNA promoter sequences defines the type of genes and hence

cellular functions that are being modulated. In the case of STAT3, the main cellular functions being regulated are cell survival and proliferation, as well as regulation of stromal reactions (eg, angiogenesis, immune evasion) for malignant tumours.²² STAT3-inducible genes involved in these regulatory functions are survivin, cyclin-D1, Bcl-xl, Mcl-1, vascular endothelial growth factor and proinflammatory cytokines.

In colorectal cancer (CRC), STAT3 expression has recently been examined *in vitro*^{23,24} and *in situ*.^{25,26} These studies suggest a frequent up regulation and activation of STAT3 protein in CRC, whereby phosphorylated (P-)STAT3 expression was considerably up regulated in the progression of adenoma to carcinoma and correlated with histopathological classification of primary adenocarcinomas. Moreover, Ma *et al*²⁵ found an association of STAT3 and cyclin D1 protein expression in CRCs. However, a detailed evaluation of STAT3 mRNA and protein expression in CRC cells and its effect on mRNA and protein levels of STAT3-inducible targets, such as survivin, cyclin D1, Bcl-xl and Mcl-1, has not been performed so far. In view of the potential novel application of STAT inhibitors, a better understanding of the STAT3 pathway in normal and cancerous tissues of patients with CRC is essential to keep up the discussions of the direct actions of STAT inhibitors *in situ*.

We have therefore characterised STAT3 mRNA and protein expression in matching normal and tumour tissues from 32 patients with CRC and correlated this with mRNA and protein

Abbreviations: CRC, colorectal carcinoma; QRT-PCR, quantitative reverse transcriptase polymerase chain reaction; STAT, signal transducers and activators of transcription

Table 1 Histopathological characteristics of colorectal cancer specimens

T category	n	N category	n	Grading	n
1	1	0	21	G1	1
2	2	1	7	G2	28
3	24	2	4	G3	3
4	5				

expression of the STAT3-inducible targets—namely, survivin, cyclin D1, Bcl-xl and Mcl-1. For evaluation of mRNA levels, we applied quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) to microdissected normal epithelial and invasive carcinoma cells. In addition, serial tissue areas of those used for mRNA measurements were used for the preparation of tissue microarrays and immunohistochemistry for UP-STAT3 and P-STAT3 protein expression as well as for all selected STAT3-inducible targets. Finally, STAT3, survivin, cyclin D1, Bcl-xl and Mcl-1 levels were correlated with tissue morphology and tumour classification. Correlation of STAT3 levels with those of STAT3-inducible genes allowed for the identification of direct interactions of STAT3 with its targets in CRC tissues.

MATERIALS AND METHODS

Tissues

Formalin-fixed and paraffin-wax-embedded tissues were selected from 32 patients (19 men and 13 women) with colon cancer (archive, Pathologisches Institut Freiburg, Freiburg, Germany; ethical approval 251/04, Ethik-Kommission, Albert-Ludwig-Universität Freiburg, Freiburg, Germany). The median age of patients at surgery was 64.5 (range 29–90) years, and none of the patients had received neoadjuvant radiotherapy or chemotherapy.

After reclassification of the resection specimens according to the World Health Organization criteria,²⁷ representative tissue blocks were selected for QRT-PCR analysis and immunohistochemistry (tissue microarrays; Beecher Instruments, Sun Prairie, USA). Table 1 summarises the histopathological data of the resection specimens (all pMx). For control purposes, tissues of normal and malignant tissues of the breast, ovary and lymph nodes were included in the immunohistochemical analysis.

Microdissection and QRT-PCR

For mRNA isolation, 10-µm-thick sections were cut from each tissue block, and normal colonic epithelial cells or invasive carcinoma cells were dissected using fine needles. Total RNA extraction was performed using a proteinase K-mediated lysis followed by phenol–chloroform/isopropanol purification.^{28, 29} RNA pellets were diluted in water and measured using the Nanodrop system (ND1000, Peqlab, Erlangen, Germany).

For cDNA synthesis, 1.5 µg of total RNA was transcribed using the MMLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) using random primers (Roche Diagnostics, Mannheim, Germany) and yeast tRNA as carrier.

QRT-PCR was performed for STAT3, survivin, cyclin D1, Bcl-xl and Mcl-1 mRNA expression using the ABI7000 system (Appliedbiosystems, Weiterstadt, Germany); table 2 lists the primers and probes. Primers and probes were selected to be intron spanning and to be preferentially located in the mRNA region coding for the protein sequence targeted by the antibodies used for immunohistochemistry (STAT3). The validation parameters for QRT-PCR included primer and probe optimisation, determination of PCR efficiency, sequencing of the PCR products as well as several negative controls: (1) RNA without reverse transcription, (2) cDNA synthesis using water and (3) PCR analysis with

Table 2 Primer and probes used for quantitative reverse transcriptase polymerase chain reaction

Gene name	Primers and probes (5'–3')	Amplicon size (bp)
STAT3	F-gccagagagccaggagca R-tgaagctgacccaggtagcgctgc P-acacagataaactggcttcagggtatg	75
Cyclin D1	F-cctgacggcgcgagaag R-aggttcacitgagctgttcac P-agggagctgctgcaatggagctg	103
Survivin	F-ggcccagtggttcttctgctt R-gcaaccggacgaatgcttt P-agccagatgacgacccatagaggaaca	91
Bcl-xl	F-aatgaccactagagccttgga R-agggagaacggcgctgggatactttgt P-tgctgcatgttcccatagagt	78
Mcl-1	F-gacaaacgggactggctagt R-cctctaggctctctacatggaga P-acaaagaggctgggatgggttgagg	80

F, forward; P, probe; R, reverse.

Sequences for the forward and reverse primers as well as the FAM-TAMRA fluorescently labelled P are shown with corresponding amplicon sizes.

water. The QRT-PCR assays for the housekeeping gene TBP and the markers survivin and cyclin D1 have been already described previously,^{29, 30} with TBP being a reliable housekeeping gene for mRNA expression analysis in colonic tissues.³¹

For the study samples, cDNAs of matching normal and tumour cells were analysed in the same QRT-PCR run for all markers, and mRNA levels were quantified using the Ct-values in the formula 2^{-ddCt} .³²

Immunohistochemistry

For immunohistochemical analysis, tissue microarrays (spot diameter 0.2 µm) were prepared from serial tissue of the area used for microdissection and QRT-PCR analysis.

Sections, 4 µm thick, were cut from the tissue microarrays for immunohistochemistry of STAT3 (clone 124H6, Cell signalling technologies, NEB, Frankfurt, Germany, 1:800), P-STAT3 (tyr705; Cell signalling, 1:20), cyclin D1 (clone DCS-6, DakoCytomation, Glostrup, Denmark, 1:50), survivin (Novus Biologicals, Acris Antibodies, Hiddenhausen, Germany, 1:100), Bcl-x (clone NC1, NovoCastra, LabVision, Newmarket, UK, 1:20), Mcl-1 (clone 38G3, NovoCastra, 1:100) and Ki-67 (clone MIB-1; DakoCytomation; 1:50). In brief, tissue sections were deparaffinised and antigen retrieval was performed by either microwave treatment in citrate buffer pH 6 (STAT3, P-STAT3, survivin, Bcl-x) or pressure cooker-mediated antigen retrieval in TRIS buffer pH 9 (Mcl-1, cyclin D1, Ki-67). Primary antibodies were incubated for 30 min (cyclin D1, Mcl-1), 60 min (STAT3, survivin) or overnight (P-STAT3, Bcl-x), followed by secondary antibody (goat anti-mouse or anti-rabbit; DakoCytomation) for 60 min.

Table 3 Comparison of mRNA and protein expression between normal colonic epithelium and invasive carcinoma cells (χ^2 test)

Marker	mRNA	Protein (epithelium)	
		Cytoplasmic	Nuclear
STAT3	0.200	STAT3: 0.023 P-STAT3: 0.006	STAT3: 0.161 P-STAT3: 0.871
Cyclin D1	<0.001	0.758	<0.001
Survivin	<0.001	0.173	0.173
Bcl-xl	<0.001	0.55	NA
Mcl-1	0.637	0.733	NA
Ki-67	NA	NA	<0.001

NA, not applicable; P, phosphorylated; STAT3, signal transducers and activators of transcription 3.

Figure 1 Summary of mRNA levels in normal colonic epithelium (No) and invasive colorectal carcinoma (Tu). Quantitative reverse transcriptase polymerase chain reaction was performed, as described in the Materials and methods section, on mRNA extracted from each of the microdissected normal and invasive carcinoma cell samples of the 32 cases. mRNA levels are expressed as a relative ratio according to the formula $2^{-\Delta\Delta C_t}$. Cyclin D1, survivin and Bcl-xl mRNA levels are significantly raised in the tumour compared with normal epithelial cells. STAT3, signal transducers and activators of transcription 3.

Slides were developed using the ABC-AEC system (STAT3, P-STAT3, survivin, Bcl-x) or the LSAB/Neufuchsin system (Mcl-1, cyclin D1, Ki67) from DakoCytomation.

The scoring of stainings was semiquantitative with respect to intensity (0, negative; 1, weak positive; and 2, strongly positive) and localisation of staining (cytoplasmic v nuclear).

Statistical analysis

Statistical analysis included all patient data (age, sex), tumour information (location, T and N category, grading) and experimental data. Correlations between parameters were performed using Pearson's correlation coefficient. The t test was used to compare all continuous parameters from normal versus tumour samples, and the χ^2 test was used for comparison of discrete

parameters. All statistical evaluations were performed at the 95% level.

RESULTS

Screening of mRNA and protein expression of STAT3 and survivin, cyclin D1, Bcl-xl and Mcl-1

QRT-PCR analysis showed mRNA expression for all of the genes in normal and invasive carcinoma cells; however, marked differences in mRNA levels were seen between normal and tumour cells of the individual genes (table 3, fig 1). Significantly higher mRNA levels were observed in invasive carcinoma cells than in normal epithelial cells for cyclin D1 ($p < 0.001$), survivin ($p < 0.001$) and Bcl-xl ($p < 0.001$). By contrast, both STAT3 ($p = 0.200$) and Mcl-1 ($p = 0.637$)

Figure 2 Representative immunohistochemical stainings of the signal transducers and activators of transcription 3 (STAT3) protein. (A,B,E,F) Staining with the antibody to total (unphosphorylated) STAT3 protein. (C,D,G,H) Staining with the antibody to tyr705-phosphorylated STAT3 protein. Besides cytoplasmic staining in (A–D), nuclear tyr705-STAT3 expression can be seen in (C,D).

mRNA levels were similar in normal and neoplastic colonic epithelial cells.

Immunohistochemical analysis of all markers in serial tissue areas showed that, in contrast with STAT3 mRNA, protein expression of STAT3 was significantly higher in tumour cells than in normal cells (fig 2). This was true for both UP-STAT3 ($p = 0.023$) and P-STAT3 ($p = 0.006$) protein expression. Both nuclear STAT3 and P-STAT3 staining was not different in normal colonic epithelia and invasive carcinomas.

For STAT3-inducible targets, a significant increase occurred for nuclear cyclin D1 ($p < 0.001$) and Ki-67 ($p < 0.001$) protein staining in the tumour compared with normal (fig 3) cells, whereas survivin ($p = 0.17$), Bcl-x1 ($p = 0.55$) and Mcl-1 ($p = 0.73$) expression was similar in normal and tumour cells (data not shown).

Correlation of mRNA and protein expression of STAT3 and survivin, cyclin D1, Bcl-x1 and Mcl-1 with tumour histology and patient characteristics

To investigate whether specific mRNA or protein levels were associated with tumour classification, all markers were correlated with the T and N category as well as the differentiation grade of the tumours. Whereas no significant associations were found with respect to T category, significantly higher STAT3 ($p = 0.019$) and Bcl-x1 ($p = 0.001$) mRNA levels were seen in cases with positive ($n = 11$) than in cases with negative ($n = 21$) lymph nodes. The tumour differentiation grade was significantly associated with survivin mRNA expression ($p = 0.015$), as well as with cyclin D1 ($p = 0.027$), Mcl-1 ($p = 0.022$) and Ki67 ($p = 0.004$) protein expression. Finally, we

Figure 3 Correlation of marker expression with lymph node status. Quantitative reverse transcriptase polymerase chain reaction was performed, as described in the Materials and methods section, on mRNA extracted from microdissected invasive carcinoma cells of the 32 cases. mRNA levels are expressed as a relative ratio according to the formula $2^{-\Delta\Delta Ct}$. Significantly higher mRNA levels of signal transducers and activators of transcription 3 (STAT3) and Bcl-xl were observed in lymph node-positive (N+) than in lymph node-negative (No) cases.

found no significant associations of marker expression with age and sex of the patient or the site of tumour location (colon v rectum).

Correlation of STAT3 expression with STAT3-inducible genes

To evaluate the functional relevance of STAT3 expression for induction of specific targets, STAT3 mRNA and protein (unphosphorylated and phosphorylated) levels were correlated with mRNA and protein levels of cyclin D1, survivin, Bcl-xl and Mcl-1 (table 4).

STAT3 mRNA expression was associated with cyclin D1 ($p = 0.018$), survivin ($p = 0.003$) and Bcl-xl ($p = 0.003$) mRNA expression, as well as with nuclear survivin protein expression ($p = 0.039$).

UP-STAT3 protein expression was significantly correlated with Bcl-xl ($p = 0.024$) mRNA expression as well as with protein expression of P-STAT3 ($p = 0.014$, nuclear), cyclin D1 ($p = 0.024$, cytoplasmic), survivin ($p = 0.019$, cytoplasmic; $p = 0.049$, nuclear) and Bcl-xl ($p = 0.001$, cytoplasmic).

Cytoplasmic staining of P-STAT3 correlated with survivin protein expression ($p < 0.001$, cytoplasmic) and nuclear P-STAT3 staining ($p = 0.014$). Nuclear staining of P-STAT3 was also associated with survivin protein expression ($p = 0.010$, nuclear), but was further linked to Bcl-xl ($p = 0.038$) and Ki-67 ($p = 0.017$) staining.

DISCUSSION

This study is the first to have analysed in detail the mRNA and protein expression of both STAT3 and four STAT3-inducible

Table 4 Correlation of STAT3 expression with STAT3-inducible targets in colorectal tumour cells

Marker	STAT3 mRNA	STAT3 protein		P-STAT3 protein	
		cyt	nuc	cyt	nuc
Cyclin D1 mRNA	0.018	0.024 (cyt)			
Survivin mRNA	0.003	0.019 (cyt)		0.001 (cyt)	0.001 (nuc)
Survivin protein	0.039 (nuc)	0.049 (nuc)			
Bcl-xl mRNA	0.003	0.024			0.038
Bcl-xl protein		0.001			
Mcl-1 mRNA			0.05		
Ki-67 protein					0.017

cyt, cytoplasmic; nuc, nuclear; STAT3, signal transducers and activators of transcription.

targets—namely, cyclin D1, survivin, Bcl-xl and Mcl-1—in invasive CRCs. Using both QRT-PCR and immunohistochemical analysis of STAT3 and the four targets, we found that the only clear-cut association was a significant correlation between STAT3 and Bcl-xl expression at the mRNA and protein levels, as well as their significant correlation with lymph node status in CRCs. Also, survivin expression was closely linked to STAT3 mRNA and protein levels. By contrast, cyclin D1 and Mcl-1 seem to be markedly up regulated in CRCs, irrespective of STAT3 protein expression. This is most probably linked to the proliferative activity, as shown by our concomitant analysis of Ki-67. Our findings show that STAT3, Bcl-xl and survivin are candidate molecular markers for further testing of their prognostic or predictive value in CRC, especially in patients with lymph node metastasis. In view of novel therapeutic agents targeting the STAT3 pathway,^{14–16–19} we have provided data that are of relevance for future clinical studies on STAT-targeted treatment strategies in CRC.

The STAT3 pathway is involved in the control of transcription of several cell-cycle and proliferation-associated genes,^{1–2} and has been shown to be differentially regulated in a variety of malignancies in humans.^{4–5} One concept of this pathway is that activated STAT3 undergoes a series of phosphorylation steps that enables STAT3 to dimerise within the cytoplasm, translocate to the nucleus and induce transcription of specific genes.²⁰ The phosphorylation of tyr705 and ser727 residues in the STAT3 molecule is therefore exploited by *in situ* and *in vitro* tests to determine the active function of STAT3. However, a recent *in vitro* study showed that UP-STAT3 may also be active and functional.²¹ Especially in transformed cells, this may represent a mechanism of constitutive “activation”. In our study, we have therefore used an *in situ* approach that permitted the correlation of total and P-STAT3 expression (cytoplasmic or nuclear) with the expression of STAT3-inducible targets at the mRNA and protein levels. For this we used formalin-fixed and paraffin-wax-embedded CRC tissue specimens. Although there is some concern about the use of such tissues for analysis of protein phosphorylation,³³ phosphorylation-specific antibodies for *in situ* analysis have been developed in recent years and numerous studies have shown the reliability of P-STAT3 antibodies in fixed specimens.^{7–8–26} Also, quantitative analysis of mRNA levels by RT-PCR is reliable from such fixed tissues.^{28–30}

Our data on STAT3 protein expression confirmed the results of previous studies,^{8–11–12} showing that STAT3 protein expression is markedly increased in invasive epithelial tumours compared with that in normal epithelium, and therefore represents a specific target for novel treatment strategies. Interestingly, this marked increase in STAT3 protein expression was not paralleled by an increase in STAT3 mRNA expression. This may indicate that on transformation to invasive (or metastasising) tumour cells, the normal regulation of STAT3 protein expression and deactivation is upset, which could occur independent of STAT3

mRNA levels. In fact, constitutive STAT3 expression and sequestering of STAT3 protein in granules has been reported.²¹ A recent study on STAT3 protein expression in CRC²⁶ showed that increased P-STAT3 staining was seen in 69 of 95 colorectal adenocarcinomas, and STAT3 expression levels correlated with tumour and venous invasion, lymph node metastasis and Union International Contre le Cancer stage. The correlation of STAT3 protein expression with the lymph node status of resected CRC is further supported by these results, whereby cases with positive lymph nodes (T1–4; N1, 2) showed higher STAT3 protein levels in the primary tumour than cases without lymph node involvement (T1–4; N0). Further evaluation of metastatic lesions (lymph node or distant) with respect to STAT3 expression should clarify in more detail the apparent role of the STAT3 pathway for tumour metastasis.

Irrespective of the effect of STAT3 protein expression on the metastasising potential of CRCs, correlation of STAT3 expression with the levels of STAT3-inducible targets yielded a close link between high STAT3 levels and induction of Bcl-xl and survivin expression. The direct link between STAT3 and Bcl-xl expression has been recently shown *in vitro*,²⁴ whereby inhibition of STAT3 resulted in the down regulation of Bcl-xl, cell-cycle arrest and apoptosis. We have now shown that the close association between STAT3 and Bcl-xl also occurs in a representative cohort of CRCs, supporting the further evaluation of STAT3 inhibitor-driven tumour cell apoptosis as a novel treatment strategy in patients with CRC. Interestingly, not only STAT3 but also Bcl-xl expression was associated with lymph node involvement in our series of cases, further supporting a specific role of STAT3-induced Bcl-xl expression for tumour metastasis.

In summary, we showed that both UP-STAT3 and P-STAT3 expression are markedly increased in invasive CRCs. This is associated with Bcl-xl and survivin induction and increased proliferation, as well as with lymph node metastasis. Our study therefore provides the basis for further examination of the prognostic or predictive value of these molecular markers in CRC.

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