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Interferon-alpha treatment may negatively influence disease progression in melanoma patients by hyperactivation of STAT3 protein

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ABSTRACT

Interferon-alpha (IFN-alpha) is an important drug used in anti-melanoma therapy. However, metastases eventually reappear in almost 60% of melanoma patients, who have received adjuvant cytokine therapy suggesting that IFN-alpha can paradoxically promote disease progression in some cases, at least. In this study, we have investigated the possibility that a growth-promoting STAT3 protein might be activated by interferon-alpha in melanoma cells. We examined 24 primary cultures established from node metastases of melanoma patients who were monitored in a 5-year clinical follow-up. The patients differed in the course of disease and survival end-points. Using Western blot analyses, we show that interferon-alpha stimulated STAT3 phosphorylation at tyrosine (Y705) residue in 17% of cases. These over-reactive cell populations originated from patients who had the shortest disease-free intervals. A significant correlation was obtained between the length of survival end-points and a lack of STAT3 activation by IFN-alpha. No STAT3 induction was observed in normal melanocytes. The STAT1 activation at tyrosine (Y701) occurred at a similar frequency as that of STAT3 (17%) albeit in different patients, no clear correlation with the clinical status could be made. The interferon-alpha/beta receptors (IRFARs) were expressed irrespective to the signal transducers and activators of transcription (STATs) inducibility suggesting that signalling defects occur downstream from IRFAR. We propose that in some cases the application of IFN-alpha could increase the probability of disease progression via overactive STAT3. The tests for STAT3 inducibility prior to cytokine immunotherapy in the clinic are therefore warranted.

1. Introduction

Normal growth and differentiation of cells depend on a continuous flow of signals from their environment. One important set of signals is provided by cytokine family comprising over 40 glycoproteins, including interleukins (ILs), interferons (IFNs), tumour necrosis factors (TNFs) or growth factors (GFs). They have been defined well both by their structure and by target receptors they bind to. Of these cytokines, IFN-alpha has been used in the clinic as an anti-cancer agent in

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leukaemias and solid tumours, including malignant melanoma. The rationality of using IFN-alpha for the management of melanoma stands on its well-known resistance to chemotherapy and/or radiotherapy. It may exert a direct antiproliferative and proapoptotic activity, and may inhibit tumour-induced neoangiogenesis.[1,2] Interferon-alpha of its own could also have a role in the aetiology of melanoma since its promoter appears to be frequently inactive in melanoma cell lines.[3] However, it is believed that the antitumour activity of IFN-alpha is mainly accomplished via stimulation of host immune system including up-regulation of the expression of adhesion molecules and MHC class I antigens.[4–6] Despite its undisputed anti-cancer properties, various clinical trials have demonstrated that the response rate of melanoma patients treated with IFN-alpha was less than expected to be, and the reason has been a topic of frequent debates. The validity of adjuvant IFN-alpha administration for melanoma treatment has also been questioned in a recent study.[7] One explanation is that the IFNs could exert differential effects on immune system and tumour itself.

Recent molecular studies of events that follow IFN receptor binding and operate downstream from receptor to target genes defined number of proteins involved in the intracellular signalling cascade. Of those, receptor-associated Janus tyrosine kinases (JAKs), signal transducers and activators of transcription (STATs), suppressors of cytokine signalling (SOCSs) and protein inhibitors of activated STATs (PIASs) represent key proteins that ensure execution of biological effects of most, if not all cytokines.[8–10] Since STAT proteins besides transducing signals from many ligands act as transcription factors modulating the expression of genes that control cell growth, differentiation, apoptosis and tumour angiogenesis, it is not surprising to note that various perturbances in these proteins are frequently found in human cancer cells and tissues. Of seven members of STAT family recognised so far the particularly noticeable cancer-associated changes such as constitutive expression, down-regulation and aberrant phosphorylation were mainly detected in STAT1 and STAT3 proteins.[8,11] Both proteins show similar DNA-binding specificities; however, they also exert distinct patterns of binding to natural enhancer elements, which may explain differences in their cellular functions..[12,13] STAT1 behaves as a tumour suppressor, acting as an apoptosis inducer and negative regulator of cell growth and angiogenesis.[14,15] We have recently shown that malignant melanoma associates with deficient IFN-induced STAT1 phosphorylation, and that the absence of STAT1 phosphorylation at tyrosine 701 (Y701) in response to IFN-gamma in *ex vivo* melanoma cells positively correlates with disease outcome in melanoma patients.[16,17] STAT3 has opposing biological effects and its constitutively active (DNA binding) variant induces malignant transformation and oncogenesis, i.e. it functions as oncogene.[18,19]

A considerable amount of data on the pathophysiology of STAT1/STAT3 transcription factors including cancer-associated alterations has been accumulated. However, little is known about the association of their abnormal expression and activation with the clinical parameters, i.e. disease outcome, metastatic potential and treatment response. In the current study, we investigated the activation response of STAT3 to IFN-alpha and gamma in melanoma cells derived from node metastases of 24 melanoma patients and evaluated the possible association of phosphorylation responses with the course of disease. We have obtained a positive correlation between the absence of STAT3 activation by IFN-alpha and a better prognosis in melanoma patients.

2. Materials and methods

2.1. Patients

Twenty four melanoma patients of clinical stages II and III (UICC TNM classification, the fifth edition) were included in this study. After regional lymph node dissection for metastatic disease, patients underwent postsurgical adjuvant immunotherapy with IFN-α2b (Schering-Plough, Kenilworth, NJ) according to the following regimen: an initial induction phase of 10 MU subcutaneously (s.c.) daily for 5 days during 4 weeks followed by a maintenance dosage of 10 MU s.c. three times a week for 48 weeks. The basic characteristics of the sample are shown in Table 1. The median follow-up was 54 months. The course of the disease as from the first diagnosis is illustrated in Fig. 1. For the comparisons of laboratory data with the disease evolution of each patient, the following intervals were employed: 1. disease-free survival (DFS), i.e. months elapsing from the initial diagnosis until the first relapse (metastases in the regional lymph nodes); 2. progression-free survival (PFS), i.e. interval from the first relapse till the subsequent progression; 3. overall survival (OS), i.e. months from the first diagnosis until the exitus and/or termination of the study. At the stage of lymph node metastases the regional lymph node exenteration was performed, and all nodes were examined by histology. The melanoma cells separated from nodes with massive tumourous infiltration were used for *in vitro* STAT3 activation studies.

2.2. Melanoma cell culture

The infiltrated lymph node was cut into small pieces, gently minced and the monocellular suspension was obtained by repeated pipetting. The cells were seeded into Petri dishes and grown in DMEM with glutamine (PAN Biotech GmbH, Aidenbach, Germany) supplemented with 15% fetal bovine serum (FBS; PAN Biotech GmbH, Aidenbach, Germany), insulin

Table 1 – Basic characteristics of the patients.

Parameter	Value
Sample	N = 24
General characteristics[a]	
Age (years)	56 (36; 71)
Breslow score	2.9 (1.5; 5.5)
Risk localisation in upper body parts	70.8%
Clark score ⩾ 4	87.5%
Median of survival end-points	
Disease-free survival (DFS)	19.6 months
Progression-free survival (PFS)	44.6 months
Overall survival (OS)	54.3 months

a Quantitative variables summarised as median and 5–95% percentiles (in parentheses).

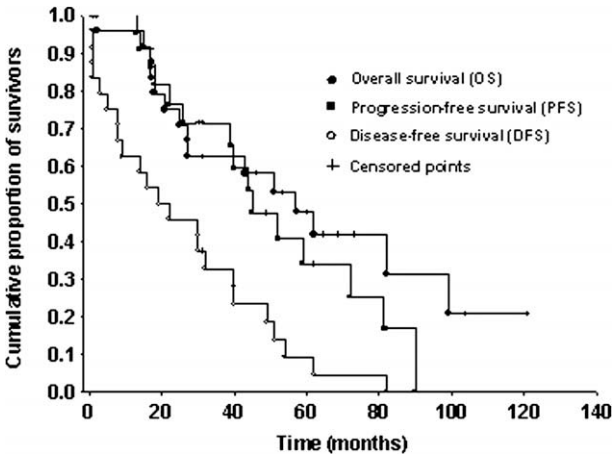

Fig. 1 – Classification of patient samples according to the course of the disease.

(5 ng/ml) and antibiotics. To verify the nature of growing cells prior to the activation experiments, the cells grown on slides were fixed and immunostained using monoclonal antibodies (Melan-A and Tyrosinase, Novocastra; S-100 and HMB-45, BioGenex), which are considered as melanoma phenotypic markers. Only those cultures showing positivity to at least one immunoreagent in more than 90% of cells were used for the study (see Fig. 2). The activation experiments were performed with cultures that were maintained *in vitro* for a maximum of four weeks. Subconfluent cells were serum starved overnight in DMEM before exposure to IFNs. Activation dosages of either IFN were selected from dose–response curves. IFN-alpha and IFN-gamma were used at concentrations of 5000 IU/ml and 10 ng/ml, respectively. Cells were incubated with IFNs for 30 min at 37 °C, and control samples were incubated in parallel without IFN treatment.

2.3. *Growth inhibition assay*

The antiproliferative effect of IFN-alpha was assessed in four primary cell cultures. Cells were seeded in duplicates into 96-well plates at a cell density of 2,000 cells per well. One day after seeding, the medium was replaced by medium containing IFN-alpha at concentrations: 1000, 5000 and 20,000 U/ml. After 3 days of culture, WST-1 colorimetric assay (Roche; Mannheim, Germany) was performed according to the manufacturers' protocol. The percentage of growth inhibition was calculated in relation to the growth of untreated control cells. Each experiment was repeated three times.

2.4. *Reagents and antibodies*

Recombinant human IFN-alpha and IFN-gamma were purchased from Sigma (St. Louis, United States of America (USA)). For the detection of STAT3 protein, rabbit polyclonal antibody against C-terminal domain of STAT3 (C-20) as well as mouse monoclonal antibody recognising STAT3 protein (F-2) was employed (Santa Cruz Biotechnology, California, USA). For the detection of STAT3-phosphorylated forms, anti-pY705 rabbit polyclonal antibody (Cell Signalling Technology, Beverly, USA) and anti-pS727 rabbit polyclonal antibody (AbCam, Cambridge, United Kingdom (UK)) were used. STAT1-phosphorylated forms were detected with anti-pY701 rabbit polyclonal antibody (Cell Signalling Technology, Danvers, MA, USA) and anti-pS727 monoclonal antibody (PSM1[16]). Mouse monoclonal antibody against the R1 chain of the IFN-alpha/beta receptor (R&D systems, USA) was used.

2.5. *Western blot analyses*

The protein content in whole-cell extracts (WCEs) was determined by means of Bradford assay (Bio-Rad, Munich,

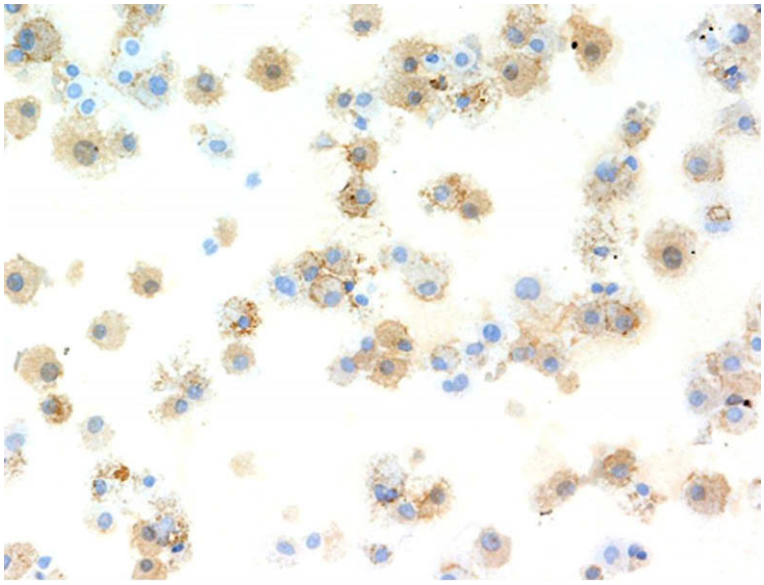

Fig. 2 – A typical culture of melanoma cells established from lymph node metastasis. Immunoperoxidase staining with Melan-A antibody (original magnification: 200×). Note, a highly homogeneous population of cells showing positive immunoreaction and malignant morphology.

Germany). Supernatants were incubated overnight at 4 °C with polyclonal antibody C-20 (dilution 1:100) and protein A – Sepharose CL-4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitates were washed four times with ice-cold Frackleton buffer. The beads were eluted by boiling in Laemmli sample buffer for 5 min. Equal amounts of proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% bovine serum albumin (BSA) in TBS buffer containing 0.1% Tween-20 (TBST) and incubated with the predetermined concentrations of specific antibodies. For the dilution of anti-STAT3 antibodies, TBST supplemented with 5% BSA (Sigma, St. Louis, USA) and 0.05% NaN_3 was employed. After washing, the blots were incubated with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham, UK) and were developed using the enhanced chemiluminescence (ECL) detection system (Amersham, UK) according to manufacturers' instructions. Signals were quantified by densitometry using ImageQuant software (GE Healthcare, USA). Phosphorylated STAT levels were normalised to total STAT signals.

2.6. RNA analysis

Total RNAs were isolated using RNeasy kit (Qiagen, Hinden, Germany) followed by the DNase I treatment (TURBO DNA-free, Applied Biosystems/Ambion, Austin, TX, USA). The cDNAs were prepared by reverse transcription of RNAs with Superscript II Reverse Trancriptase (Invitrogen, Carlsbad, CA, USA) and amplified using the PCR Arrays master mix and IFNAR2, STAT3 and GAPDH primers, respectively (SABiosciences, Frederick, MD, USA) according to the manufacturers' recommendation. The primers amplified a 153 bp fragment of the IFNAR2 gene encoding a R2 chain of the interferon-alpha/beta receptor. The 10-μl aliquots were loaded on 1.5% agarose gels, stained by ethidium bromide and visualised using a CCD camera (Ultralum, Claremont, CA, USA).

2.7. Statistical analysis

All statistical tests were performed based on intention-to-treat principle, and all failure or death events were binary recorded as fully equivalent. A value $p < 0.05$ was taken as a universal limit for statistical significance. Standard descriptive statistics were used to describe primary data (median, 5–95% percentiles, frequency tables). Stratified Kaplan–Meier product-limit method was applied to discriminate survival rates between two or more subgroups. Peto-Prentice generalised log-rank test was applied as comparative statistical test.

3. Results

3.1. Immunohistochemical characterisation of primary melanoma cell cultures and cytotoxic effect of interferons

Primary cell cultures were established from 24 regional node melanoma metastasis after the surgery. After one month of *in vitro* growth, the cells were phenotyped using antibodies against antigens, which were known to be expressed in skin neuroectodermal cells. It is evident that immunostaining of cells with an antibody against Melan-A resulted in uniform staining patterns indicating homogeneity of cell populations (Fig. 2). All cultures had mostly single cell phenotype.

Cytotoxic effect of interferon-alpha was assessed in several cell populations by counting of viable cells at 24, 48 and 72 h intervals. There was about 10–40% growth inhibition depending on the experiment and the line. Examples of growth curves are shown in Fig. S1.

3.2. Western blot analysis of STAT3 phosphorylation

Antibodies against STAT3 phosphoforms (pY705 and pS727) were used for the detection of activated STAT3 known to be associated with cancer phenotype. Information on the drug dosage effect was obtained by analysis of STAT3 phosphorylation levels in two selected primary cultures (Fig. 3A). It is evident that the LM48/2 line in the left panel showed the absence of STAT3 induction at low (1×10^3 U/mL), while there was a significant induction at both higher (5×10^3 and 20×10^3 U/mL) concentrations of IFN-alpha. In contrast, the VJ22 line in the right panel showed a relatively strong basal STAT3-pY signal, which was little (IFN-gamma) or not (IFN-alpha) increased after the cytokine treatment.

The concentration of IFN-alpha of 5×10^3 U/mL was selected for further experiments involving Western blot analyses of 24 primary cultures. Immunostaining of protein blots with an antibody against an amino acid determinant (Fig. 3B and C, STAT3 – total), indicated the expression of STAT3 protein in all samples with little differences in signal intensities. In contrast to non-phosphorylated STAT3, there were differences in the basal levels of phosphorylated STAT3 isoforms between the samples. For example, the HM68 and LM58 patients had low STAT3-pY levels, while both PT56 and SJ97 patients showed strong pY-STAT3 signals (Fig. 3B) consistent with the reported variation in basal phosphorylation levels of STAT3 in melanoma patients.[20,21] Based on the STAT3-pY/STAT3-total ratio we can assess that the basal STAT3-pY levels were relatively low in melanocytes compared to most melanoma samples.

The interferon treatments resulted in enhanced STAT3-pY and STAT3-pS signals in some, but not in all samples (Fig. 3B and C and summarised in Table S1). Based on the inducibility of phosphorylated Y705-STAT3 isoforms, the 24 melanoma samples could be divided into three groups (Table 2). In the first group (13 cases/54%), no activation with either interferon was observed. The second group comprises samples with STAT3 induction after IFN-gamma but not alpha (7 cases/29%). Normal melanocytes fell into this group. The third group (4 cases/17%) comprises samples showing induction of STAT3 phosphorylation with both IFNs. There were no cases of STAT3 induction with IFN-alpha without concomitant activation with IFN-gamma. It is necessary to stress that the experiments were carried out using cells within the 1 month following surgical excision for cell culture (the first passage). Further passages and prolonged cultivation of cells resulted in altered STAT3-pY responses in some cases (Ludmila Lauerova – unpublished).

The relation between STAT3 activation response at pY705 induced by IFNs and disease outcome is demonstrated in

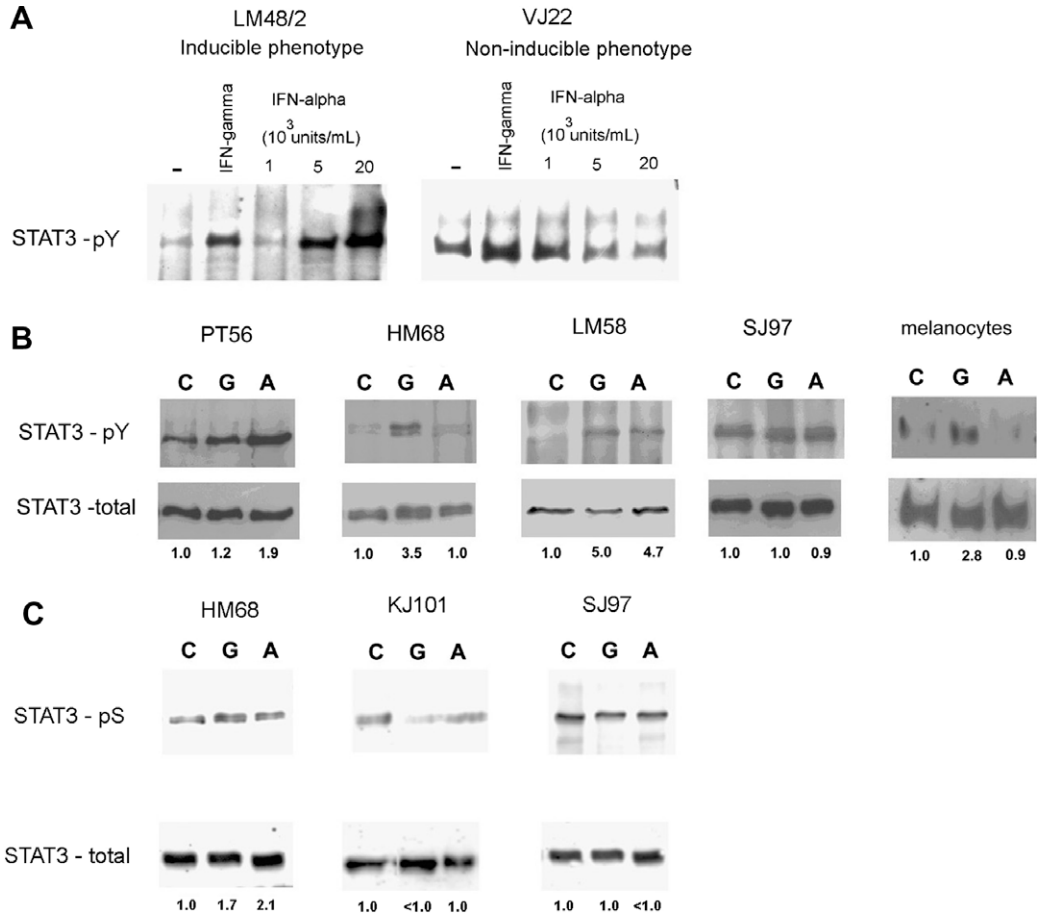

Fig. 3 – STAT3 expression and activation in melanoma cell populations. (A) Dosage effect of IFN-alpha on stimulation of STAT3 phosphorylation at Y705. (B) Example of immunoblotting analysis of STAT3-pY705. (C) Example of immunoblotting analysis of STAT3-pS727. C, control; G, interferon-gamma; A, interferon-alpha. STAT3-pY, staining with anti phosphotyrosine (pY705) antibody. STAT3-pS, staining with anti phosphoserine (pS727) antibody. STAT – total, staining with an antibody to the primary amino acid determinant. Exposure of chemiluminescent bands to the film was 1–5 min. Signals were evaluated by densitometry and expressed as a STAT3-p/STAT3-total ratio (numbers below each lane). For control samples, the ratios were arbitrarily chosen as 1.0.

Table 2 – STAT3 activation in response to IFN-alpha/-gamma in melanoma patients.

	IFN-alpha		IFN-gamma	
	Not inducible[a] Cases/percentage	Inducible Cases/percentage	Not inducible Cases/percentage	Inducible Cases/percentage
pY705	20/83	4/17	13/54	11/46
pS727	22/92	2/8	22/92	2/8

a The data were taken from Western blot analyses (Fig. 3 and Table S1). Samples showing >20% increase of pY705 and pS727 signal intensities over non-treated controls were considered as 'inducible'.

Table 3. Patients disclosed as activation responders to IFN-alpha, i.e. whose *ex vivo* metastatic melanoma cells showed IFN-alpha-induced STAT3 phosphorylation at Y705 exhibited a significantly shorter disease-free survival (DFS), progression-free survival (PFS) and overall survival (OS) as compared to the non-responder group. These analyses provide evidence that the activation of STAT3 at pY705 induced by IFN-alpha negatively correlates with the disease outcome.

In contrast to phosphorylation at tyrosine, only two samples showed an increased phosphorylation at serine 727 residue (pS727) after IFN-alpha or IFN-gamma stimulation (Fig. 3C and Table 2). However, the activation response at pS727 in these patients did not uniformly reflect disease evolution since one case was lost after 51 months due to dissemination, whereas the other was surviving 62 months without progression.

Table 3 – Survival end-points in relation to different measures of STAT3 activation.

Parameter[a]	DFS[b]	PFS[b]	OS[b]
Induction by IFN-alpha: Not inducible (N) activity versus inducible (I) activity			
STAT3 at Y705	$p = 0.049$ N: 34.9 month I: 5.0 months	$p = 0.041$ N: 62.3 month I: 26.1 months	$p = 0.039$ N: 78.4 month I: 26.5 months
Induction by IFN-gamma: Not inducible (N) activity versus inducible (I) activity			
STAT3 at Y705	$p = 0.693$ N: 18.0 months I: 23.8 months	$p = 0.496$ N: 39.2 months I: 43.9 months	$p = 0.726$ N: 40.2 months I: 55.4 months

a Activation of STAT3 at Ser 727 is not analysed due to a very low frequency of inducible cases ($N = 2$).
b Significance level of log-rank test comparing the two variants (p) and median values of survival in months.

3.3. *Western blot analysis of STAT1 phosphorylation*

The STAT1 phosphorylation status at Y701 residues was assessed in the same sets of patient cell populations as in the STAT3 analysis. Examples of immunodetections with antibodies against total and phosphorylated STAT1 are shown in Fig. 4, and summarised in Tables 4 and S1. In the first group (4 cases/17%), no activation with either interferon was observed. The second group comprises samples with STAT1 induction after IFN-gamma but not alpha (16 cases/66%). The third group (4 cases/17%) comprises samples showing induction of STAT1 phosphorylation with both IFNs. In general, STAT1 was more frequently induced with interferon-gamma (88%) than STAT3 (46%). However, the basal levels of STAT1-pY were much lower than those of STAT3-pY suggesting constitutive activation of STAT3 (but not STAT1) in tumour cells.

3.4. *RNA and protein analysis of interferon-alpha/beta receptor (IFNAR) in melanoma cells*

Of the 24 patient samples, 18 samples (75%) did not show induction at either STAT3 or STAT1 after IFN-alpha. We thought that this might be due to deficiencies in the IFNAR expression. We therefore analysed expression of IFNAR genes in the selected samples that show (VJ 22, LM48/2) or do not (SJ 97, KJ 101) show STATs activation. At the RNA level, we studied the expression of the R2 chain of the IFNAR using RT PCR assay. A ~150 bp product was amplified with the IFNAR2-specific primers in all cDNA samples analysed including those of normal melanocytes (Fig. 5A). The signal intensities were comparable in cell populations with both inducible and non-inducible phenotypes indicating that the IFNAR2 genes were equally transcribed in melanoma cells. Similarly, at the protein level, an antibody recognising a R1 chain of the IFNAR showed immunoreactivity with all protein extracts (Fig. 5B). Thus, it appears that there were probably no defects in the expression of interferon-alpha/beta receptor among the melanoma cells.

4. Discussion

Activated STAT3 is considered as an oncogene displaying growth-promoting and anti-apoptotic properties.[22] In this study, we have analysed interferon-dependent STAT3 expression and activation in short-term melanoma cell cultures that are established from lymph node metastases. We believe that these primary cultures, in contrast to the established cell lines, probably better reflect the situation *in vivo*. Indeed, the cell populations were remarkably homogeneous expressing markers characteristic for melanoma lineages. Western blot analyses showed a constitutive expression of STAT3 protein in all the samples, which is consistent with the requirement of this molecule for survival of tumour cells in culture. The short-term stimulation with both types of interferons leads to a variable elevation of the levels of STAT3 phosphoforms. Significantly, all four cultures (17%) that showed a strong STAT3 induction after IFN-alpha treatment were derived from patients with short disease-free interval and poor survival (STAT3-pY705 inducible cohort of patients, Table 3). Perhaps, in these cases STAT3 protein might transactivate growth-promoting and/or anti-apoptotic genes leading to a more aggressive tumour phenotype. In support, *c-myc* and *JunB* oncogenes have been shown to contain STAT3-binding sites in their

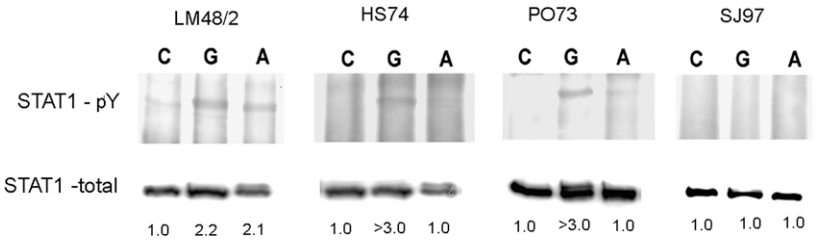

Fig. 4 – Example of immunoblotting analysis of STAT1 in melanoma cell populations and normal melanocytes. STAT1-pY, staining with anti-phosphotyrosine (pY701) antibody. The symbols are the same as those shown in Fig. 3. Note relatively weak basal levels of STAT1-pY.

Table 4 – STAT1 activation in response to IFN-alpha/-gamma in melanoma patients.

	IFN-alpha		IFN-gamma	
	Not inducible Cases/percentage	Inducible Cases/percentage	Not inducible Cases/percentage	Inducible Cases/percentage
pY701	20/83	4/17	4/17	20/83
pS727	20/83	4/17	19/79	5/21

The data were taken from Western blot analyses (Fig. 4 and Table S1).

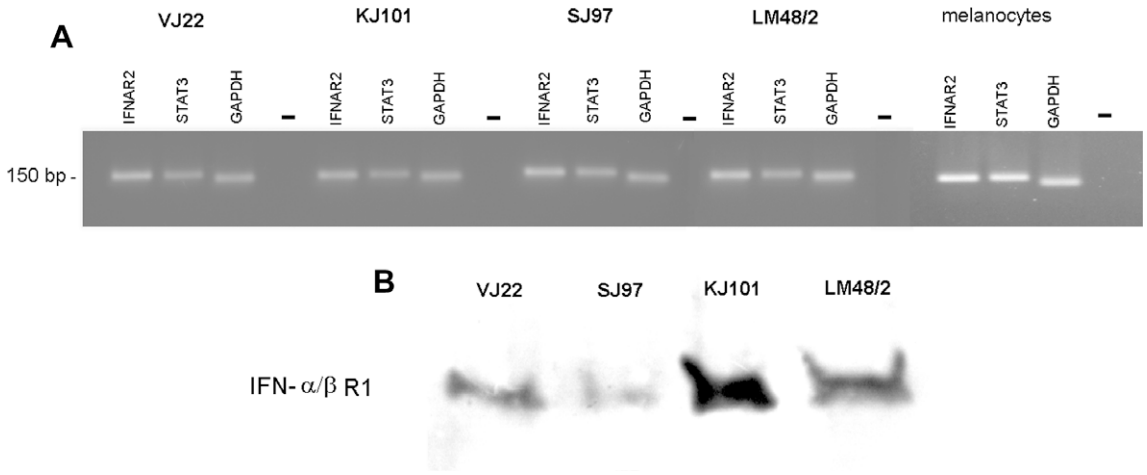

Fig. 5 – The RNA (A) and protein (B) analysis of IFNAR expression. (A) Ethidium bromide-stained gels with PCR products obtained after amplification of cDNA samples using IFNAR2, STAT3 and GAPDH – specific primers. Amplification of the GAPDH gene was used as a positive control. No products were obtained without a reverse transcription step (lane '-'). (B) Example of Western blot analysis of protein extracts using antibody against interferon-alpha/beta receptor (R1 chain).

promoters.[23] Although IFN-alpha induced a variable growth retardation (10–40%) in all the cell cultures, we were unable to observe a significant correlation between IFN-alpha resistance and STAT3 phosphorylation status. For example, both relatively sensitive LM48/2 and KJ101 lines (Fig. S1) differed in the ability to activate STAT3-pY after IFN-alpha (Table S1). This may be due to the absence of additional growth-promoting agents in cell culture medium, ongoing cell senescence in advanced passages and perhaps other unknown factors. The STAT3 inducibility with IFN-gamma was more frequent compared to that of IFN-alpha, and there seems to be a trend towards better prognosis in inducible cases; however, correlation with disease evolution showed no statistical significance (Table 3).

Impaired STAT1 signalling has also been noted in melanoma by several studies although the correlations with sensitivity towards interferons were often contradictory.[16,24–28] In our experiments, increased STAT1 phosphorylation after IFN-alpha occurred at a frequency (17%) similar to that of STAT3. The frequency of STAT1 activation is slightly lower than that previously observed in immortalised melanoma lines (37%)[16], which can be explained by selection of clones with 'overresponsive' phenotype. With the exception of the LM 48/2 patient there was no overlap between inducibilities of STAT1 and STAT3 between the cultures suggesting that both proteins are using different regulatory pathways. Basal levels of STAT3 have been inversely correlated with IFN-alpha-induced STAT1-pY in melanoma cell lines.[20] In contrast to STAT3, there was no significant correlation between STAT1 responders (to IFN-alpha) and clinical state (Table S2).

4.1. *STAT3 regulatory pathways in melanoma cells*

Several lines of evidence indicate that activation of STAT3 by IFN-alpha may occur somewhat aberrantly in a subset of tumours. Firstly, interferons in contrast to interleukin 10 do not seem to be strong activators of STAT3.[29] Secondly, binding of STAT3 to DNA was not stimulated by interferon-alpha in melanoma cell lines.[30] Finally, in a mouse model system, STAT3 was activated by IFN-gamma only in the absence of functional STAT1.[31] Failure of STAT3 activation could be explained by an inefficient expression of interferon receptor. However, all cell populations analysed showed the expression of interferon-alpha/beta receptor at both RNA and protein levels. These results are partially consistent with the recent immunohistochemical study showing the expression of the receptor in melanoma cells.[21] In addition, there were cases of alternative induction of STAT1 and STAT3 molecules (LM 58, RJ 61, DM 90 and VJ22). Together, it seems that though the functional defects or variation in IFNAR expression levels (Fig. 4) cannot be entirely excluded, it is more likely that negative regulatory pathways involving suppressors of cytokine signalling (SOCS) and proteins that inhibit activated STATs (PIAS) might be involved. In this context, most melanoma cell

lines, in contrast to normal melanocytes, failed to activate SOCS3 following IFN-alpha suggesting that SOCS3 could play a role.[32] On the other hand, constitutively expressed SOCS3 has been negatively correlated with cell sensitivity to IFN-gamma.[33,34] It will be interesting to determine the integrity of negative regulatory systems of JAK/STAT signalling in tumours and tumour-derived cultures.

4.2. *Possible clinical implication of STAT3 activation by interferon-alpha*

Metastatic disease will eventually develop in almost 60% of the melanoma patients, who have received adjuvant IFN-alpha therapy.[35] In addition, the overall response rate to IFN-alpha in the stage of metastatic disease is just 15–20%. Thus, there appears to be a distinct subset of melanoma patients, who will respond favourably to IFN-alpha. A similar speculation on the existence of subgroups of melanoma patients responding differently to IFN-alpha in the clinic was reported by Lens.[36] Our data suggest that this subset may comprise tumours that do not hyperactivate STAT3 after the IFN-alpha treatment. Conversely, metastatic disease could be promoted in tumours that do show a strong STAT3 activation phenotype. Significantly, the STAT3-inducible phenotype was observed in primary cultures of melanoma cells obtained from individuals who failed IFN-alpha immunotherapy.[20] It is also noteworthy that STAT3 activity was increased in human brain metastatic melanoma cells when compared with primary melanoma specimens.[37] The generality of our findings will require confirmation on a larger series of cases and other types of tumours.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.01.009.

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