Lack of STAT 1 phosphorylation at TYR 701 by IFNγ correlates with disease outcome in melanoma patients*

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Received December 16, 2004

STAT 1, a member of signal transducer and transcription activator family has been implicated as key downstream mediator of interferon (IFN) signaling. Its functional activation requires phosphorylation at Tyr 701 and Ser 727 residues. Various STAT abnormalities have been found in cancer cells but their relation to oncogenesis, tumor behavior and disease outcome remains mostly unknown. We have examined the inducibility of STAT 1 phosphorylation by IFN α /γ in primary cultures established from melanoma lymph node metastases at first progression and correlated our results with disease outcome and overall survival. Forty-four patients at clinical stage I–III at initial diagnosis entered the study. STAT 1 inducibility of phosphorylation by IFNs was assessed in melanoma cell lysates by means of standard immunoprecipitation and Western blotting using polyclonal and monoclonal antibodies. Lack of STAT 1 phosphorylation at Ser 727 after either IFN was recorded in 75% of patients, however, no correlations with disease evolution could be proved. In contrast, STAT 1 phosphorylation response at Tyr 701 after IFNα occurred in 13 (29.5%) and after IFNγ in 32 (73%) patients. Inducibility of STAT 1 activation at Tyr 701 but not at Ser 727 driven by IFNγ but not by IFNα significantly and favorably influenced disease-free interval and overall survival. In conclusion, these results show that the absence of IFNγ inducibility of STAT 1 phosphorylation at Tyr 701 positively correlates with disease outcome in malignant melanoma patients and may represent new independent prognostic marker.

Key words: STAT 1 activation, interferons, malignant melanoma, disease prognosis

Signal transducers and activators of transcription (STATs) are multigene family of latent cytoplasmic proteins that function as important downstream mediators of a number of extracellular signaling molecules including cytokines, growth factors, hormones and oncoproteins [2, 11]. It has been well established that various members of STATs are critical in maintaining cellular homeostasis. Seven members of STAT proteins so far recognized have been structurally characterized and the function of various domains and regions that take part in the processes of signal-induced activation, transduction and DNA binding was determined [7, 15]. The essential molecular events that follow ligand-receptor interactions comprise tyrosine phosphorylation of receptor by receptor-associated Janus tyrosine kinases (JAKs), thereby creating receptor docking sites for recruitment of cytoplasmic STAT proteins. Upon association with phosphorylated receptor, STATs are activated by phosphorylation on conserved tyrosine residues and homodimerize or heterodimerize through SH2 domain-phosphotyrosine interactions. Activated STATs translocate into nuclei, bind to recognition sequences in the promoters of specific cellular genes and modulate their transcription [6, 15, 21]. Recent investigation on the mechanisms regulating STAT-mediated transcriptional power revealed that phosphorylation of STATs at conserved serine residues also actively operates in signaling pathways enhancing transcriptional potential mediated by activated STATs and probably acting as a dephosphorylation signal [7, 11, 13, 15]. Molecular state and the activity of STATs are con-

*The study was supported in part by grants NC/7139-3 from the Internal Grant Agency of the Czech Ministry of Health, 301/03/0370 from the Grant Agency of the Czech Republic and by Projects MZO 00209805 and MSM 6198959216.

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trolled through their interactions with a number of cytoplasmic and nuclear proteins including variety of transcription factors as well as by STATs mutual crosstalking [7, 15]. Although there is a close homology among the individual STAT members, they differ in their response to the particular dominant ligand and consequently in the downstream target gene clusters that are transcribed. In general, STATs 1 and 2 principally mediate *in vivo* response to interferons (IFNs), STAT 3 represents main target for IL-6, STAT 4 and STAT 6 as dominant mediators of IL-12 and IL-4 control Th cell differentiation, whereas STAT 5 in a form of its two isoforms is indispensable for growth hormones and prolactin [14]. However, there are frequent overlaps within STAT responses to dominant ligands as well as responses to additional ligands outside dominant ones. As mediators of a broad range of external stimuli and modulators of gene expression, STATs play important role in regulating cell cycle, proliferation, differentiation, senescence and apoptosis. Perturbances in STAT proteins have been described in several human pathological conditions including immune or developmental disorders and cancer.

Down-regulated expression or constitutive release of STATs and their inappropriate activation are the most frequent alterations observed in many human malignancies [2, 3, 10, 17]. Reports on the oncogenic activity of persistently activated STAT 3 [4] together with abnormalities in STAT activation observed in some malignant cells [12, 17, 20] support a notion that STAT dysregulation is involved in the biology of cancer and its responsiveness to cytokine-based therapy [5]. To our knowledge, little is known whether cancer-associated STAT deregulations described in *in vitro* model systems operate also *in vivo* situations, and whether some of them might have a link with the biological behavior of the tumor and disease outcome.

We have examined the inducibility of STAT 1 phosphorylation in primary cultures of melanoma cells derived from regional lymph node metastases and exposed *in vitro* to IFNα and IFNγ, respectively, and correlated the abnormalities with disease outcome and overall survival in 44 melanoma patients. Our results revealed significant positive correlation between lack of inducibility of STAT 1 phosphorylation at Tyr 701 by IFNγ and disease outcome. Since the possible bias due to the influence of known risk factors has been excluded, we recognized that the absence of IFNγ inducibility of STAT 1 phosphorylation at Tyr 701 in melanoma cells may represent new independent positive prognostic marker in melanoma patients. This prognostic influence was proved in multivariate Cox regression models taking both overall survival and early development of the disease as principal endpoints.

Material and methods

Patients. Forty-four patients with malignant melanoma at clinical stage I-III (UICC TNM classification, the 5th edition, 1997) without node metastases at the time of initial diagnoses (median age 56 years) entered the study. The basic characteristics of the sample are in Table 2. The median follow-up was 61 months. At first disease progression mostly to regional lymph nodes, the metastases were surgically dissected and patients were further treated according to standard protocols. Primary cultures from metastatic lymph node of each patient were established and examined for the inducibility of STAT 1 phosphorylation at tyrosine (Y 701) and serine (S 727) residues, respectively, after exposing the melanoma cells to either interferon gamma (IFNγ) or interferon alpha (IFNα). The phosphorylation response of STAT 1 was correlated with the following variables: time interval from initial diagnosis to first progression (EFS_1), disease-free survival from date of first progression to date of subsequent relapse (EFS_2), survival from the first progression to date of death (S_p), overall survival from the initial diagnosis to the time of last follow-up control or date of death, respectively (OS).

Melanoma cell cultures. Melanoma cells were derived from a biopsy of lymph node massively infiltrated by metastatic melanoma cells. The biopsy was cut into small pieces, gently minced and the monocellular suspension obtained by repeated pipetting. The cells of forty-four samples were grown in medium DMEM with L-glutamine (PAN Biotech GmbH, Germany) supplemented with 10% FBS (PAN Biotech GmbH, Germany), insulin (5 ng/ml) and antibiotics. To verify the nature of growing cells prior to activation experiments, the cells grown on slides were fixed and immunostained using monoclonal antibodies (NCL-L-MelanA, Novocastra; NCL-L-Tyrosinase, Novocastra; HMB 45, BioGenex) which are considered as melanoma phenotypic markers. Only those cultures showing positivity to at least one immunoreagent in more than 80% of cells were used for the study. Primary cultures were maintained *in vitro* for a maximum of four weeks.

Reagents and antibodies. Recombinant human IFNα and IFNγ were purchased from Sigma (USA). For the detection of STAT 1 protein and its phosphorylated forms, we developed polyclonal antiserum against C-terminal domain of STAT 1 (S1C) as well as monoclonal antibodies recognizing STAT 1 protein (SM1) and its S 727 phosphorylated form (pSM1). Polyclonal antibody anti-PY 701 STAT 1 (Sigma, USA) was used for analysis of IFN-induced STAT 1 activation.

STAT 1 phosphorylation analysis. The method was described previously [1, 12]. Briefly, IFNγ was used at a concentration of 10 ng/ml and IFNα at concentrations of 1000 IU/ ml and 5000 IU/ml, respectively. Cells were incubated with IFNs for 30 min at 37 °C. Control samples were without IFN treatment. Induction of STAT 1 phosphorylation at Y 701 and S 727 was assessed in cellular lysates by means of Western blots.

Cellular lysates, immunoprecipitation and Western blot. Cell lysis, immunoprecipitation and Western blotting were carried out by standard methods as described elsewhere [1,

12]. Cells were lysed for 5 min on ice in Frackleton buffer. Protein concentration was determined by Bradford assay (Bio-Rad, Germany). For immunoprecipitation, polyclonal antibody S1C and protein A-Sepharose beads were used (Amersham Pharmacia Biotech, Sweden). For Western blot analyses, antibodies SM1, pSM1 and anti-PY 701 were employed.

Statistical analyses. All statistical tests were performed on intention-to-treat principle, no case was excluded prior to the analyses and all failure events or death events were recorded as fully equivalent. A value $\alpha<0.05$ was taken as a universal limit for statistical significance in all univariate and multivariate analyses. Although the study investigated maximum of attainable cases in common consequential clinical recruitment, it might be limited in sample size. Therefore, namely outcomes of multivariate analyses are presented as pilot estimates that need further confirmation in independent studies. Standard descriptive statistics was used to express differences among subgroups of cases (mean supplied with 95% confidence limits or relative frequencies). Standard univariate statistical techniques were used to test differences between chosen subgroups of patients: Fisher's exact test in binary outcomes, ML chi-square test for ordinal categorical variables, unpaired Student's t-test for normally distributed continuous variables and Mann-Whitney test for non-normally distributed continuous variables. Stratified Kaplan-Meier product-limit method was applied to discriminate survival rates between two or more subgroups. Standard Peto-Prentice generalized log-rank test was used as comparative statistical test. Both univariate and multivariate analytic strategies were applied to quantify predictive power of examined variables to predefined study endpoints: overall survival and event-free survival to the 1st progression. All potential predictors were coded as binary factors according to their risk values and then processed in univariate and multivariate Cox regression models. A stepwise multivariate Cox proportional hazard analysis was used as final model identifying significant predictors of event-free or overall survival. Hazard ratio was estimated with appropriate 95% confidence limits and supported by significance level. The final set of independent prognostic factors was identified by backward stepwise selection algorithm.

Results and discussion

Table 1 summarizes results of STAT 1 activation in IFN-treated and untreated 44 melanoma patients. Intensity of signals was compared with reference signals of controls present at the identical membrane. Basic statistical description of the investigated sample including common follow-up characteristics of disease development illustrates Table 2. Follow-up period (median 61 months) was sufficiently long for stratified survival analysis

Table 1. STAT 1 phosphorylation in IFN α/γ treated and untreated primary cell cultures derived from melanoma patients

Malignant melanoma patient (n = 44)	Inducibility					
	PS 727 STAT 1			PY 701 STAT 1		
	IFN α	IFN γ	untreated	IFN α	IFN γ	untreated
1	I	I	+	I	I	+
2	I	I	+	I	I	+
3	I	N	+	I	I	+
4	I	N	+	I	I	+
5	I	N	–	I	I	–
6	N	N	–	N	N	+
7	N	N	+	N	N	+
8	N	N	+	N	N	+
9	N	N	+	N	N	+
10	N	N	+	N	N	+
11	N	N	+	N	N	+
12	N	N	+	N	N	+
13	N	N	+	N	N	+
14	N	N	+	I	I	+
15	N	N	+	I	I	–
16	N	N	+	I	I	–
17	N	N	+	I	I	–
18	ND	ND	ND	I	I	–
19	I	I	+	N	N	+
20	I	I	+	N	N	–
21	N	N	+	N	I	–
22	N	N	+	N	I	+
23	N	N	+	N	I	+
24	N	N	+	N	I	+
25	N	N	+	N	I	–
26	N	N	+	N	I	+
27	N	N	+	N	I	+
28	N	N	+	N	I	–
29	N	N	+	N	I	–
30	N	N	+	N	I	–
31	N	N	–	N	I	–
32	N	N	–	N	I	–
33	N	N	+	N	I	–
34	N	N	+	N	I	–
35	N	N	+	N	I	–
36	ND	ND	ND	N	I	–
37	I	N	+	N	I	+
38	N	I	–	N	I	+
39	N	I	+	N	I	–
40	N	I	+	I	I	+
41	N	N	+	I	N	+
42	I	I	–	N	I	+
43	I	I	–	N	I	–
44	I	N	+	N	N	+
Percentage of non-responders	73.8%	78.6%		72.7%		27.3%

I – inducible, N – not inducible, ND – not detected.
Untreated: +: positive signal, –: negative signal.

Table 2. Basic characteristics of the sample and disease development

Parameters	Values
Patients	
N	44
Gender (male)	63.6 %
Age at diagnosis (years)[1]	56 (36; 76)
Follow-up time (months)[1]	61 (24; 114)
Primary tumor - localization	
Trunk, head, neck	59.1 %
Other	40.9 %
Primary tumor – Breslow	
Summary statistics [1]	2.4 (1.0; 9.4)
Category: Breslow < 1.0	11.7 %
Category: Breslow > 3.0	35.3 %
Primary tumor – Clark	
Category: Clark ≤ III	35.7 %
Category: Clark > III	64.3 %
First disease progression	
Event-free survival (EFS_1 in months)[2]	21 (4; 44)
Distant metastases in liver, brain or lungs	N = 21 (47.7 %)
Survival after first disease progression	
Event-free survival (EFS_2 in months)[2]	3 (1; 7)
Survival after first progression (S_P in months)[2]	18 (8; 35)
Overall survival (OS in months)[2]	55 (20; 125)

[1]Summary statistics: estimate of median supplied with 10% and 90% percentiles (in parentheses). [2]Median survival time estimated on the basis of Kaplan-Meier analysis supplied with 25% and 75% percentiles (in parentheses). For detailed description of survival parameters see legend in Table 2.

and all examined survival endpoints reached median level. Four principal survival criteria were defined in order to map different phases of disease evolution, i.e. time from primary diagnosis to first progression and subsequent phases of event-free survival or survival. Overall survival (OS) covered all these episodes and could be considered as dominant integrating endpoint of the study.

Stratified survival analyses as performed by standard Kaplan-Meier method are displayed in Table 3. We have found that EFS_1 as an indicator of early risk development as well as OS significantly coincide with the most risk factors, what is in accordance with data reported elsewhere. In contrast, detailed analyses focused on disease development after 1st progression showed no significant associations. Event-free intervals and survival after 1st progression appeared to be unpredictable on the basis of factors examined and listed in Table 3. Age, risk location of primary tumor and Breslow score were recognized as potential risk factors both for EFS_1 and OS, what is in agreement with known data and complying with known melanoma prognostic indices. Furthermore, early risk development of disease (distant metastases into the vital organs, first progression up to 12 months from diagnosis) significantly contributed to the risk load and negatively influenced overall survival.

To assess IFN inducibility of STAT 1 phosphorylation at Tyr 701 and Ser 727 as the potential predictors of disease evolution, the activation response was determined in patient samples and related to the disease outcome of individual patients (Tab. 4 and 5). The frequency of STAT 1 phosphorylation at Ser 727 was more or less the same for IFNα and IFNγ (11 vs. 9 inducible cases) and both STAT 1 activating signals led to dominant proportion of non-responders (33 vs. 35 cases). On the other hand, there were significant differences between IFNα and IFNγ induction of STAT 1 phosphorylation at Tyr 701 (13 vs. 32 inducible cases – see Tab. 4). Thus, IFNγ provided significantly increased proportion of inducible responders (73%), what corresponds with the reports describing IFNγ as the principal activator of STAT 1 [18].

Analyses and statistical evaluation of the relationship between inducibility of STAT 1 activation in melanoma cells by either IFN and selected phases of disease evolution revealed the most important and apparently so far not recognized result of the study (Tab. 5). Inducibility of STAT 1 activation at Tyr 701 but not at Ser 727 driven by IFNγ but not by IFNα significantly influenced early event-free development of the disease and overall survival as well. The group of patients whose malignant cells were lacking IFNγ inducibility of STAT 1 phosphorylation at Tyr 701 had better prognosis with respect to event-free and overall survival intervals as compared to the group of responders.

Figure 1 clearly demonstrates that activation response of STAT 1 at Tyr 701 after IFNγ significantly decreases median of EFS1 and OS.

As the inducibility of STAT 1 phosphorylation by IFNγ at Tyr 701 appeared to be a meaningful predictor of shorter survival, we had to verify its dependence/independence on the other known risk factors. Association analyses as summarized in Table 6 proved that IFNγ-induced STAT 1 activation at Tyr 701 can be regarded as independent on nearly all risk factors, except for sex and Clark categories. However, higher proportion of activation responders in female patients can hardly explain the prognostic potential of STAT 1 activation since sex categories themselves have very limited influence on disease development.

The potential contribution of inducibility of STAT 1 phosphorylation to the prediction of disease development was also confirmed in univariate and multivariate time-to-event regression models that excluded potential bias due to masking influence of other risk factors. All potential risk factors first entered univariate Cox regression models. The analyses in Table 7 confirmed previously recognized patterns, namely significantly increased relative risk in cases having inducible STAT 1 at Tyr 701 by IFNγ and similar behavior of risk categories of age, Breslow score and location of primary tumor. Risk parameters describing early disease development (distant metastases at time of 1st progression

Table 3. Survival endpoints stratified according to potentially important risk factors

Stratifying parameters	Survival endpoints[1]			
	EFS_1	EFS_2	S_P	OS
	Median survival time and statistical tests			
Age at diagnosis				
Age ≥ 50 years	18	3.5	14	34
Age < 50 years	48	2.5	18	63
Statistical significance	p = 0.035	p = 0.572	p = 0.368	p = 0.029
Sex				
Men	20	3	15	38
Women	28	4	16	55
Statistical significance	p = 0.146	p = 0.980	p = 0.948	p = 0.311
Primary tumor: locality				
Trunk – neck – head	15	2	14	27
Other	30	4	16	48
Statistical significance	p = 0.046	p = 0.588	p = 0.239	p = 0.038
Clark				
Clark ≤ III	25	7	15	41
Clark > III	27	3	18	53
Statistical significance	p = 0.918	p = 0.299	p = 0.685	p = 0.597
Breslow				
Breslow ≥ 3	16	2	12	24
Breslow < 3	31	4	17	44
Statistical significance	p = 0.046	p = 0.592	p = 0.077	p = 0.039
1st progression				
Distant metastases: lung, liver, brain	20	1	13	32
No metastases in lung, liver, brain	23	6	20	44
Statistical significance	p = 0.448	p = 0.029	p = 0.047	p = 0.033
1st progression				
EFS_1 ≤ 12 months	–	2	11	14
EFS_1 > 12 months	–	4	18	58
Statistical significance	–	p = 0.337	p = 0.043	p = 0.019

[1]Survival endpoints estimated on the basis of Kaplan-Meier analysis (median of survival time). Statistical comparison of two strata: log-rank test. EFS_1 – event-free survival calculated from date of primary diagnosis (and surgically dissected tumor) to the first progression of the disease; parameter related to the primary therapy of tumors (all cases recruited to the study passed through first relapse or progression of primary disease).
EFS_2 – event-free survival calculated from date of first progression of the disease to date of subsequent risk event (survivors without risk event were censored at time of last follow-up control). S_p – survival reached after first progression of the disease calculated from date of first progression to date of death (survivors were censored at time of last follow-up control). OS – overall survival calculated from date of diagnosis to date of death (survivors were censored at time of last follow-up control).

Table 4. IFN inducibility of STAT 1 phosphorylation at Tyr 701 and Ser 727

STAT 1 phosphorylation	Responders (inducible)	Non-responders (not-inducible)	Overall difference in inducibility by IFNα or IFNγ[1]
Tyr 701			
IFNα	29.5 % (n = 13)	71.5 % (n = 31)	p = 0.013
IFNγ	72.7 % (n = 32)	27.3 % (n = 12)	(IFN a < IFN g)
Ser 727			
IFNα	25.0 % (n = 11)	75.0 % (n = 33)	p = 0.819
IFNγ	20.5 % (n = 9)	79.5 % (n = 35)	(IFN a = IFN g)

[1]Binomial test comparing inducibility (responsiveness) to IFNα and IFNγ as quantitative test of difference between these two variants.

Table 5. Survival endpoints stratified according to IFN inducibility of STAT 1 phosphorylation

Stratifying parameters	Survival endpoints[1]			
	EFS_1	FS_2	S_P	OS
	- Median survival time and statistical tests -			
ACTIVATION BY IFNα				
IFNα activation at Tyr 701				
Inducible	21	6	14	32
Non-inducible	25	3	15	44
Statistical significance	p = 0.487	p = 0.157	p = 0.669	p = 0.446
IFNα activation at Ser 727				
Inducible	21	3	14	46
Non-inducible	22	3	16	39
Statistical significance	p = 0.456	p = 0.959	p = 0.663	p = 0.696
ACTIVATION BY IFNγ				
IFNγ activation at Tyr 701				
Inducible	16	3	12	25
Non-inducible	45	4	27	80
Statistical significance	p = 0.018	p = 0.904	p = 0.112	p = 0.027
IFNγ activation at Ser 727				
Inducible	18	2	18	26
Non-inducible	24	3	14	44
Statistical significance	p = 0.449	p = 0.429	p = 0.805	p = 0.876

[1]Survival endpoints estimated on the basis of Kaplan-Meier analysis (median of survival time). Statistical comparison of two strata: log-rank test. For detailed description of survival endpoints see Table 2.

Table 6. Inducibility of STAT 1 phosphorylation at Tyr 701 in relation to the other risk factors

Risk factors and their categories	Inducibility (in % of cases)	Statistical test[1]
Age at diagnosis		
Age ≥ 50 years	72.2	P = 0.996
Age < 50 years	73.1	
Sex		
Men	43.8	P = 0.004
Women	89.3	
Primary tumor: locality		
Trunk – neck – head	61.1	P = 0.183
Other	80.7	
Clark		
Clark ≤ III	70.0	P = 0.956
Clark > III	66.7	
Breslow		
Breslow ≥ 3	69.6	P = 0.740
Breslow < 3	76.2	
1st progression		
Distant metastases: lung, liver, brain	64.3	P = 0.261
No metastases in lung, liver, brain	87.5	
1st progression		
EFS_1 ≤ 12 months	71.4	P = 0.998
EFS_1 > 12 months	76.9	

[1]Test for association between response to IFNγ and particular risk factor (Fisher's exact test).

and 1st progression up to 1 year) influenced very significantly profile of overall survival.

All potential predictors entered multivariate Cox regression models with outcomes summarizes Table 8. As expected from Table 7, EFS_1 up to 12 months occupied most significant position among independent risk predictors of OS. In addition, occurrence of distant metastases, age higher than 50 years and inducibility by IFNγ at Tyr 701 were applied as significant contributors to the final risk prognosis. Although limited in sample size, the objective multivariate analyses suggested potential role of IFNγ at Tyr 701 inducibility as independent predictor of long-term survival and early risk development of the disease as measured by event-free survival to the first progression (EFS_1) (Tab. 8).

In spite of numerous reports describing abnormal expression and/or activation of STAT proteins in various malignant cells, the important question, whether at all and to what extent STAT deregulation associates with or affects disease evolution in patients, has not been satisfactory elucidated. In this context, WIDSCHWENDTER et al [19] on the basis of examination of STAT 1 activation in archival biopsies of primary breast cancer patients demonstrated that high STAT 1 activation in primary tumor has direct link with favorable outcome of disease and can be considered as a significant indicator of good prognosis. These data are in sharp contrast with our results. However, both studies were carried out on different types of cancer and it is known that various STATs exert distinct activity in histologically different cell systems utilizing diverse activating ligands. For example, it has been shown that the outcome of STAT 1 and STAT 3 activation can be positive or negative depending on the stimulus and cell type involved [16]. Moreover, the different methodological approach, i.e. examination of STAT 1 phosphorylated form levels in biopsies versus inducibility of STAT 1 phosphorylation by exogenous IFNγ, might also account for divergent results in both studies.

It is noteworthy that in our study prevailing number of melanoma patients were lacking STAT 1 activation by IFNγ but in spite of this STAT 1 activation defect they still had better prognosis comparing to the responders. The finding that high numbers of melanoma samples were unresponsive to IFNγ could indicate that melanoma cells acquire, at certain stage of disease, growth advantage if the IFNγ/STAT 1 signaling is turned off. Such a phenotypical change is consistent with a phenomenon called immunoediting, i.e. a positive selection of cancer cells that acquire the ability to escape recognition by immune system [8, 9]. Our data, however, illustrates that immunoediting is not apparently the only mechanism affecting the be-

Table 7. Predictive value of potential risk parameters in univariate Cox regression models[1]

Parameter [2]	Predefined endpoint			
	Overall survival (OS)		Event-free survival to 1st progression (EFS_1)	
	Relative risk (95% CI)	p value	Relative risk (95% CI)	p value
Basic characteristics of patients and disease				
Age ≥ 50 years	2.39 (1.22; 4.71)	0.029	2.08 (1.08; 4.00)	0.027
Male sex	1.97 (0.81; 4.79)	0.118	1.82 (0.92; 3.51)	0.105
Breslow ≥ 3	1.34 (1.02; 1.79)	0.042	2.04 (1.02; 4.09)	0.039
Clark > III	0.88 (0.35; 2.22)	0.789	0.86 (0.43; 1.75)	0.698
Primary tumor locality: trunk-neck-head	2.27 (1.14; 4.51)	0.039	1.51 (1.02; 2.23)	0.048
STAT 1 activation by IFN α/γ at Tyr 701 or Ser 727: inducible cases				
IFN α activation at Tyr 701	0.64 (0.28; 1.46)	0.299	0.69 (0.36; 1.36)	0.295
IFN α activation at Ser 727	0.89 (0.37; 2.15)	0.798	0.69 (0.33; 1.42)	0.294
IFN γ activation at Tyr 701	2.77 (1.15; 6.70)	0.029	2.38 (1.32; 4.92)	0.013
IFN γ activation at Ser 727	1.18 (0.44; 3.20)	0.742	1.52 (0.72; 3.24)	0.291
Disease development				
1st progression with distant metastases (lung, liver, brain)	2.83 (1.23; 6.52)	0.011		
Time to 1st progression: EFS_1 ≤ 12 months	10.31 (3.51; 30.29)	<0.001		

[1]OS and EFS_1 were selected as endpoints that appeared to be significantly associated with several risk factors (see also Tab. 2). [2]All parameters were coded as binary factors according to specified risk values. EFS_1 – event-free survival calculated from date of diagnosis to the first progression (see also Tab. 2).

Table 8. Results of the multivariate stepwise Cox regression modeling[1]

End-point parameters included	Coefficient (SE)	Model log-likelihood	Log-likelihood ratio test	Relative risk[2]
Model for overall survival (OS)				
Null model		–81.5		
Step 1. EFS_1 (≤ 12 months)	1.917 (0.434)	–71.6	0.019	6.80
Step 2. + Meta	1.322 (0.312)	–65.7	0.003	3.75
Step 3. + Age (≥ 50 years)	0.912 (0.309)	–63.8	0.001	2.49
Step 4. + Ind IFN γ at Tyr 701	0.813 (0.337)	–60.3	< 0.001	2.25
Model for time to the first progression (EFS_1)				
Null model		–125.7		
Step 1. Age (≥ 50 years)	0.751 (0.289)	–120.1	0.036	2.12
Step 2. + Ind IFN γ at Tyr 701	0.885 (0.277)	–115.2	0.004	2.42

[1]Multivariate stepwise procedure was driven only by statistical measures (Log-likelihood function). [2]Relative risk associated with variables entered in multivariate models as independent predictors. EFS_1 – event-free survival calculated from date of diagnosis to the first progression (see also Tab. 2). Meta – distant metastases in liver, lung or brain at time of 1st progression. Ind IFNγ at Tyr 701 – IFNγ inducibility of STAT 1 phosphorylation at Tyr 701.

havior of the tumor and disease outcome. This study demonstrates that tumor cells, which lack responsiveness to IFNγ, have less devastating effects on melanoma patients' health resulting in a significantly better disease prognosis. It is well known that tumor environment, local immune reactions, inflammatory processes and epigenetic factors play important role in tumor growth, invasiveness and metastatic potential. It is entirely possible that these factors are favorable for melanoma prognoses if the tumor cells display aberrant responses to IFNγ. Hypothetically, it can not be ruled out that STAT 1 activation insufficiency might also negatively affect some other growth promoting exogenous signals utilizing STAT 1 pathways with consequence in the diminishing tumor cell growth.

Altogether, our findings show an unexpected-to-edged role of IFNγ signaling in development and outcome of malignant melanoma and illustrate that the lack of STAT 1 activation by IFNγ inversely correlates with disease evolution in malignant melanoma patients and may represent new prognostic marker.

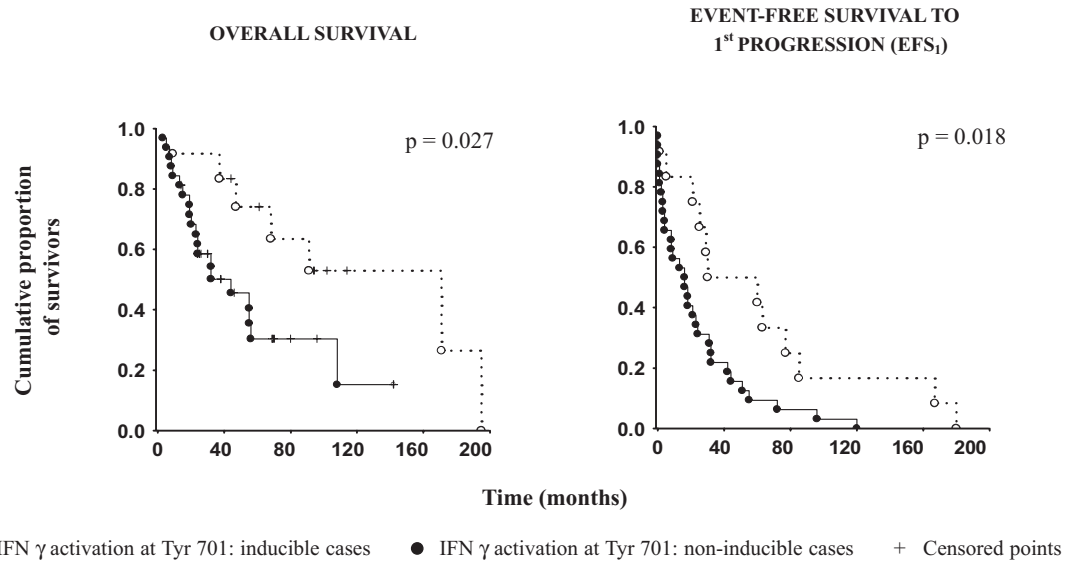

Figure 1. Kaplan-Meier analysis of survival endpoints stratified according to IFN gamma inducibility of STAT 1 phosphorylation at Tyr 701 (Log-rank test. The figure demonstrates that response of STAT 1 at Tyr 701 after IFM gamma significantly decreases median of EFS1 and OS.

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