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# BIRC6 (APOLLON) is down-regulated in acute myeloid leukemia and its knockdown attenuates neutrophil differentiation

Anna M Schläfli<sup>1</sup>, Bruce E Torbett<sup>2</sup>, Martin F Fey<sup>1,3</sup> and Mario P Tschan<sup>1,3\*</sup>

# Abstract

Background: Inhibitors of apoptosis (IAPs) were intensively investigated in the context of cancer where they promote tumor growth and chemoresistence. Overexpression of the IAP BIRC6 is associated with unfavorable clinical features and negatively impacts relapse-free survival in childhood acute myeloid leukemia (AML). Currently, BIRC6 levels in adult primary AML have not been compared to the expression in normal myeloid cells. Thus, we compared for the first time BIRC6 levels in adult primary AML patient samples to normal myeloid cells and studied its regulation and function during neutrophil differentiation.

Findings: We found significantly lower BIRC6 levels in particular AML subtypes as compared to granulocytes from healthy donors. The lowest BIRC6 expression was found in CD34<sup>+</sup> progenitor cells. Moreover, BIRC6 expression significantly increased during neutrophil differentiation of AML cell lines and knocking down BIRC6 in NB4 acute promyelocytic leukemia (APL) cells significantly impaired neutrophil differentiation, but not cell viability.

**Conclusion:** Together, we found an association of low *BIRC6* levels with an immature myeloid phenotype and describe a function for BIRC6 in neutrophil differentiation of APL cells.

Keywords: BIRC6, Acute myeloid leukemia, Acute promyelocytic leukemia, ATRA, Neutrophil differentiation

# Introduction

BIRC6 (a.k.a. APOLLON, BRUCE) is an exceptionally large protein of 528 kDa belonging to the family of inhibitor of apoptosis (IAP). BIRC6 contains one bacalovirus IAP repeat (BIR) domain that shows homology to the IAP Survivin. Furthermore, BIRC6 is the only IAP with an ubiquitin-conjugating domain further pointing to a particular function of this protein in the IAP family [1]. Several groups reported that BIRC6 executes its function via inhibition of Smac and Caspase-9 [2, 3]. Moreover, a study in breast cancer cells revealed that BIRC6 inhibits cell death by p53 destabilization and inactivation of caspase-3 [4]. The role of p53-dependent BIRC6 effector functions was



Due to their anti-apoptotic function it was hypothesized that overexpression of IAPs might contribute to tumorigenesis. Survivin, for example, one of the best studied IAPs is up regulated in most tumors and has been associated with their chemoresistence [6, 7]. Recently, Houdt et al. [8] observed BIRC6 overexpression in colon cancer stem cells when compared to more differentiated tumor cells. BIRC6 expression protected colon cancer stem cells from the cytotoxic effects of oxaliplatin and cisplatin. Furthermore, knocking-down BIRC6 led to growth inhibition in several cancer cell lines and xenografted mice and rendered the tumor cells more sensitive to 5-fluoruracil treatment in vivo and in vitro [9]. The significance of IAPs in the pathology of hematological malignancies however remains poorly understood and controversial data regarding an effect on prognosis were published. Carter et al. [10] did not find any prognostic significance for Survivin or XIAP expression in adult AML samples, whereas BIRC6



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<sup>\*</sup> Correspondence: mtschan@dkf.unibe.ch

<sup>&</sup>lt;sup>1</sup>Experimental Oncology/Hematology, Department of Clinical Research,

University of Bern, Murtenstrasse 35, CH-3010, Bern, Switzerland

<sup>&</sup>lt;sup>3</sup>Department of Medical Oncology, Inselspital, Bern University Hospital, Bern,

Full list of author information is available at the end of the article

overexpression is associated with unfavorable clinical features and negatively impacts on the 3-year relapsefree survival in childhood acute myeloid leukemia (AML) [11]. Similar results were obtained by Ismail et al. in childhood AML and acute lymphoblastic leukemia (ALL) [12]. Abe et al. [13] found lower expression of BIRC6 in bone marrow-derived cells of healthy donors compared to *de novo* AML samples.

To our knowledge, *BIRC6* levels in primary AML have never been compared to the respective expression in normal myeloid cells. Thus, we aimed at comparing *BIRC6* levels in a large cohort (n = 98) of adult AML patient samples and mature neutrophils from healthy donors. Since a hallmark of AML is a differentiation block of hematopoietic precursors at different developmental stages and since this block can be overcome by treating acute promylocytic leukemia (APL) patients with all-trans retinoic acid (ATRA) and low dose chemotherapy, we also analyzed the role of *BIRC6* during neutrophil differentiation of AML cells.

# Materials and methods

## Primary patient samples

A cohort of 98 samples from patients with a diagnosis of primary AML (FAB M0-M4) were enrolled on HOVON/ SAKK (Dutch-Belgian Hematology-Oncology/ Swiss Group for Clinical Cancer Research Cooperative group) protocols –04, -04A, -29, and –42 (available at www. hovon.nl) between 1987 and 2006 [14–18]). All patients provided written informed consent in accordance with the Declaration of Helsinki. Patient data are summarized in the Table 1. *In vitro* differentiation of CD34<sup>+</sup> progenitor cells was done as previously described [19].

# Cell lines and culture conditions

The acute myeloid leukemia (AML) cell lines HL60, FAB M2, the ATRA-resistant subline HL60-R, NB4, FAB M3 (acute promyelocytic leukemia; APL), the ATRA-resistant subline NB4-R2 and HT93 were kept in RPIMI-1640 culture media (Sigma-Aldrich, Buchs, Schweiz) containing 10 % foetal bovine serum (FBS). In order to differentiate the AML cell lines towards granulocytes 1  $\mu$ M *all*-trans retinoic acid (ATRA) was added

to the cells that were seeded at a density of  $0.2 \times 10^6$  cells/ml. Successful neutrophil differentiation was assessed by CD11b surface and by increased granulocyte colony-stimulating factor receptor (*G-CSFR; CSF3R*) mRNA expression.

# Quantitative real-time RT-PCR (qPCR) and TaqMan Low Density Array (LDA)

For RNA extraction the miRCURY RNA Isolation Kit from Exigon was used. RT-PCR has been described elsewhere [19]. Quantitative measurement of BIRC6 and *G-CSFR* mRNA was performed using the TagMan<sup>®</sup> Gene Expression assays Hs00212288 m1 and Hs00167918\_m1, respectively (Applied Biosystems, Rotkreuz, Switzerland). LDA measurements as well as data analysis were done as described [20]. HMBS primers and probes have been described previously [21]. Measurements were carried out on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland).

# Generation of BIRC6 knock-down cell lines

NB4 cells were transduced with a lentiviral vector (pLKO.1) expressing a small hairpin (sh)RNA targeting the *BIRC6* mRNA (NM\_016252.x-2281s1c1, Sigma-Aldrich, Buchs, Schweiz). Lentivirus production has been described previously [19]. As a non-targeting control we used the SHC002 lentiviral vector. Two days after transduction, NB4 cells were selected with 1.5ug/ml puromycin (Sigma-Aldrich, Buchs, Schweiz) for one week.

### Cell viability assay

For Trypan blue exclusion assay cells were washed with PBS before diluting 1:10 in 0.4 % Trypan blue solution (Sigma-Aldrich, Buchs, Switzerland). For AnnexinV staining  $1 \times 10^5$  cells were washed in 500 µl binding buffer (PBS with 0.33 g/L Ca<sup>2+</sup>) and resuspended in a final volume of 100 µl. 5 µl AnnexinV-PE were added (Immunotools, Germany) and the samples were incubated for 15 minutes at room temperature, before FACS analysis.

Table 1 AML patient characteristics from the HOVON/SAKK cohort

Cohort	Variables	Patient characteristics			FAB classification						Cytogenetics				
		Age (y)	Sex (female/male)	Total	MO	M1	M2	M3	M4	t(8;21)	inv (16)	t(15;17)	CK	NK	
HOVON/ SAKK	Range	17-74	-	-											
	Mean/median/ %	43.09/43.00 (mean/median)	59.18/40.82	100	4.08	16.33	32.65	19.39	27.55	20.41	17.35	20.41	23.47	18.37	
	No. of patients		58/40	98	4	16	32	19	27	20	17	20	23	18	

FAB, French-American-British; CK, complex karyotype; NK, normal karyotype.

## Statistical analysis

Differences between two groups were assessed using the non-parametric Mann–Whitney-U test. P-values <0.05 were considered to be statistically significant.

## Results

# Repression of *BIRC6* mRNA in AML patients with particular chromosomal aberrations

To study the BIRC6 expression patterns in normal versus leukemic myeloid cells, we measured BIRC6 levels in a large cohort of 98 primary AML patients (FAB M0-M4), in 24 granulocyte preparations from healthy donors and in 3 CD34<sup>+</sup> progenitor cell samples. We were able to detect BIRC6 in 95/98 AML patient, in 14/24 granulocytes and in 3/3 CD34<sup>+</sup> progenitor cell samples. Surprisingly, we found significantly lower BIRC6 levels in AML patients with the translocations t(8;21) and t (15;17) as well as in AML patients with a complex karyotype, whereas no significant differences in BIRC6 expression was found in AML patients with inv(16) or normal karyotype as compared to its expression levels in granulocytes from healthy donors (Figure 1). We found the lowest BIRC6 mRNA levels in in CD34<sup>+</sup> progenitor cells (Figure 1). Together, our data suggest an association of low BIRC6 expression with an immature myeloid phenotype.

# Significant *BIRC6* inductions during ATRA-induced neutrophil differentiation

In order to assess a possible role of *BIRC6* in neutrophil differentiation of AML cells, we took advantage of several AML cells line models for neutrophil



differentiation. First, we differentiated NB4 APL cells with ATRA towards neutrophils. In accordance with the low BIRC6 expression in undifferentiated AML patients compared to mature neutrophils, BIRC6 mRNA levels were up-regulated 1.8- and 3.6-fold at day 4 and 6 of ATRA treatment, respectively (Figure 2A). In order to exclude a direct effect of ATRA on BIRC6 expression, we also treated ATRA-resistant NB4-R2 APL cells with ATRA. BIRC6 transcripts were only marginally up-regulated in these cells further supporting a particular role of BIRC6 in neutrophil differentiation (Figure 2B). Neutrophil differentiation of NB4 and NB4-R2 cells was assessed by CD11b surface expression (Figure 2C). A similar induction of BIRC6 mRNA expression upon neutrophil differentiation was seen in HT93 APL cells (data not shown). In a further experiment, we determined BIRC6 expression in HL60 and ATRA-resistant HL60-R AML cells during neutrophil differentiation. We observed a significant 1.6- and 2.8fold increase of BIRC6 transcript expression at day 4 and 6, respectively. No significant change in BIRC6 expression was seen in HL60-R cells upon 4 days of ATRA treatment, whereas at day 6 a minor but significant increase was seen (Figure 2D and E). Neutrophil differentiation in HL60 cells was confirmed by CD11b FACS analysis (Figure 2F). ). Consistent with our data showing increased BIRC6 expression upon neutrophil differentiation of NB4 and HL60 AML cells, we observed a 3.3- and 2.8-fold increase in BIRC6 mRNA expression at day 3 and 6 upon in vitro granulocytic differentiation of CD34<sup>+</sup> progenitor cells, respectively (data not shown).

Overall, our findings clearly show an association of increased *BIRC6* expression with neutrophil development of AML and CD34<sup>+</sup> progenitor cells.

# Knocking down *BIRC6* attenuates neutrophil differentiation but not cell death and proliferation of APL cells

To test if specific *BIRC6* depletion inhibits neutrophil differentiation of APL cells, we generated NB4 *BIRC6* knockdown cells. *BIRC6* knockdown efficiency was confirmed in control and ATRA-treated sh*BIRC6* expressing NB4 cells (Figure 3A). Neutrophil development as determined by G-CSFR mRNA as well as CD11b surface expression was on average 50 % reduced compared to the non-targeting control transduced NB4 cells at day 4 of ATRA treatment (Figure 3B-C).

Since *BIRC6* is involved in the inhibition of apoptosis, we were asking if the observed lack of neutrophil differentiation in NB4 *BIRC6* knockdown cells is due to increased cell death in these cells. For this purpose we assessed cell death by Trypan blue exclusion and Annexin V flow cyotometry at day 4 and 6 of ATRA



treatment. We did not find any significant differences in cell viability between NB4 control and *BIRC6* knockdown cells during ATRA-induced neutrophil differentiation (Figure 3D and E). Furthermore, control transduced as well as *BIRC6* knockdown NB4 cells showed no significant differences in total cell counts at day 4 and 6 of ATRA treatment (Figure 3F).

In summary, we showed that inhibiting *BIRC6* significantly affects neutrophil differentiation of APL cells, but not cell viability.

## Discussion

In this report we publish for the first time that AML patient samples with the t(8;21), the t(15;17) or a complex karyotype express BIRC6 significantly lower than normal human granulocytes. Subtype-specific BIRC6 expression in AML is supported by earlier findings published by Ismail et al. [12]. Low expression of the anti-apoptotic IAP BIRC6 in AML may seem controversial given the often high expression of IAPs in cancer. Our findings of high BIRC6 expression in granulocytes versus AML may reflect the cellular differentiation status of these cells rather than a cancer-associated deregulation. This hypothesis is confirmed by our observation that BIRC6 mRNA levels are clearly reduced in CD34<sup>+</sup> myeloid precursor cells and increase during granulocyte differentiation. These data are in line with previous findings showing that BIRC6 is down-regulated in bone marrow-derived cells if compared to de novo AML samples [13]. Furthermore, the expression of the BIRC6 related XIAP is induced upon monocyte differentiation and contributes to monocyte cell survival. In the same study, XIAP levels declined during neutrophil differentiation supporting the cell type specific regulation of IAPs in



myeloid cells [22]. Consistent with the monocyte specific expression of *XIAP*, a correlation of *XIAP* levels with monocytic markers in AML was found [23]. In contrast to these finding in adult AML, in childhood *de novo* acute myeloid leukemia the levels of *XIAP* correlated with an immature FAB-subtype [24]. This may suggest different *XIAP* functions in distinct leukemic entities.

In conclusion, we were able to link increased *BIRC6* mRNA expression with neutrophil differentiation and inhibiting *BIRC6* resulted in attenuated neutrophil differentiation but did not alter cell survival. In summary, we established a new role for *BIRC6* in neutrophil differentiation of AML cells.

### Abbreviations

IAP, Inhibitor of apoptosis; AML, Acute myeloid leukemia; APL, Acute promyelocytic leukemia; ATRA, All-trans retinoic acid; BIR, Bacalovirus IAP repeat; ALL, Acute lymphoblastic leukemia.

### **Competing interest**

No competing interest to be declared.

### Author's contributions

AS performed the experimental research, interpreted the data and drafted the article. BET provided primary cells and essential reagents, analyzed

patient data and revised the article. MFF investigated the initial concept and experimental design and revised the drafted article. MPT designed the project and gave final approval of the submitted manuscript. All authors read and approved the final manuscript.

#### Acknowledgments

We gratefully acknowledge Dr. P.J.M. Valk and Dr. B. Löwenberg and the HOVON (Dutch-Belgian Hematology-Oncology) cooperative group for providing primary AML patient samples. Deborah Shan and Joy Chen are acknowledged for excellent technical support. This work was supported by grants from Cancer Research Switzerland KFS 02486-08-2009 (to MPT), the Bern University Research Foundation (to MPT), the Ursula-Hecht-Foundation for Leukemia Research (to MFF), the Bernese Foundation of Cancer Research (to MFF), the Werner and Hedy Berger-Janser Foundation of Cancer Research (to MFF), and the Joyce Klein Stock Gift and NIH R01HL091219 (to BET).

### Author details

<sup>1</sup>Experimental Oncology/Hematology, Department of Clinical Research, University of Bern, Murtenstrasse 35, CH-3010, Bern, Switzerland.
<sup>2</sup>Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA. <sup>3</sup>Department of Medical Oncology, Inselspital, Bern University Hospital, Bern, Switzerland.

### Received: 4 August 2012 Accepted: 16 August 2012 Published: 4 September 2012

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### doi:10.1186/2162-3619-1-25

**Cite this article as:** Schläfli *et al.: BIRC6 (APOLLON)* is down-regulated in acute myeloid leukemia and its knockdown attenuates neutrophil differentiation. *Experimental Hematology & Oncology* 2012 1:25.

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