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Epigenetic and genetic analysis of the *survivin* promoter in acute myeloid leukemia

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Abstract

Survivin, an inhibitor of apoptosis (IAP) protein plays a dual role in regulation of mitosis and inhibition of apoptosis. Survivin is expressed in embryonic and fetal organs as well as in most human cancers, but not in normal differentiated adult tissues. In this study we investigated the molecular mechanism involved in overexpression of *survivin* in acute myeloid leukemia (AML). We used methylation specific PCR (MSP) and bisulfite sequencing to analyze the methylation status of the *survivin* promoter in primary AML samples and normal peripheral blood mononuclear cells (PBMCs). Both, in patients with *de novo* AML and normal control samples an unmethylated *survivin* promoter was present. Mutational analysis of the proximal *survivin* promoter revealed three single nucleotide polymorphisms (SNPs), where the frequently occurred polymorphism (G/C) at position −31 was detectable in both, AML blasts and healthy PBMCs and showed no significant impact on prognosis in *de novo* AML patients. These results suggest that the methylation status of the *survivin* promoter and occurrence of these SNPs within the promoter region of the *survivin* gene appear to be of minor importance in leukemogenesis.

Keywords: Survivin; Transcription; Promoter; Polymorphism; DNA methylation; Acute myeloid leukemia

1. Introduction

Survivin, a member of the inhibitor of apoptosis (IAP) protein family is involved in both, inhibition of apoptosis and regulation of cell division [1–3]. It is present during embryonic and fetal development, but undetectable in normal differentiated adult tissue *in vivo* [4]. In various human cancers including acute myeloid leukemia, *survivin* is strongly overexpressed and has been established as a prognostic marker [5–8]. It is regulated in a cell cycle-dependent manner with maximal expression during G2/M phase. Survivin's cell cycle regulatory function is executed by binding several structural components of the mitotic apparatus, i.e. spindle microtubules, centrosomes or kinetochores of metaphasic chromosomes [9]. Lack of Survivin function results in disorganized mitosis and embryonal death [3,10]. The molecular mechanisms involved in the cancer-specific re-expression of *survivin* have not been completely investigated yet. Epigenetic and genetic alterations of the *survivin* promoter might have an impact on regulation of *survivin* gene expression at the transcriptional level [11]. DNA methylation is an epigenetic mechanism of gene regulation that plays a crucial role in carcinogenesis due to silencing of cancer-related genes [12]. So, hypermethylated promoters lack transcriptional activity leading to inactivation of tumor suppressor genes in human cancers [13]. On the other hand, inactivated genes, which have methylated promoter CpG islands in normal cells may be reactivated by promoter demethylation in tumor cells [13,14]. Whether the *survivin* promoter is methylated in normal cells and unmethylated and reactivated in tumor cells is still under discussion [15–18]. Furthermore, genetic alterations may also influence gene expression. Within the human *survivin* promoter, three single nucleotide polymorphisms have been identified so far [19]. The presence of the polymorphism G/C at position −31 seems to be correlated with increased expression of *survivin* [19]. Previously, it was shown that promoter elements like the cell cycle-dependent elements (CDE) and

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cell cycle homology regions (CHR) regulate the cell cycle-dependent expression of *survivin* during G2/M phase [20,21]. The polymorphism G/C at position −31 is located within the CDE/CHR repressor binding site and potentially changed the cell cycle-dependent transcription of *survivin* through the functional disruption of this motif [19].

In this study, we investigate whether the methylation status of the *survivin* promoter or polymorphisms within the promoter region are involved in leukemogenesis. We detected an unmethylated *survivin* promoter in DNA samples of *de novo* AML patients and healthy PBMCs. In addition, the common polymorphism G/C at position −31 within the human *survivin* promoter was observed with the same frequency in both, AML blasts and control samples and showed no significant impact on prognosis. Therefore, we suggest that methylation status as well as occurrence of SNP at position −31 is not involved in leukemogenesis.

2. Material and methods

2.1. Cell culture and patient samples

The human colon adenocarcinoma cell line SW48 was cultured in RPMI-1640 medium and the human cervix carcinoma cell line HeLa was grown in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) at 37 °C in 5% CO_2 in air. Both media were supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Patient-derived cell samples ($n = 59$; bone marrow $n = 29$, peripheral blood $n = 30$) were consecutively collected, Ficoll-purified (Biochrome, Berlin, Germany) and cryopreserved within the German multicentre AML-BFM93 and AML-CG92 studies. All patients had *de novo* AML. As controls, peripheral blood mononuclear cells (PBMCs) from healthy donors ($n = 6$) were isolated by Ficoll gradient centrifugation.

2.2. Bisulfite genomic modification and sequencing analysis

For bisulfite modification, 1.5 μg genomic DNA was extracted using the QIAamp Mini Kit (Qiagen, Hilden, Germany) and denatured in 1N NaOH, followed by conversion in 0.5 mM hydroquinone and 3 M sodium bisulfite under an O_2 exclusive layer of mineral oil for 16 h at 50 °C. DNA was then purified with the QIAex Kit (Qiagen), ethanol-precipitated and resuspended in H_2O. For bisulfite sequencing, primers were designed which anneal independent of methylation status: survM/Useq sense 5′-GGGTTGTTAGGTAGGGGGTAA-3′ and antisense 5′-AAACTCCAAAACTCAAATAATACTCC-3′. The PCR conditions were: 94 °C for 5 min, 94 °C for 30 s, annealing at 59 °C for 60 s, elongation at 72 °C for 60 s and a final cycle at 72 °C for 7 min. Amplificates were then purified and ligated in a pGEMTeasy vector (Promega, Mannheim, Germany). Plasmid DNA from different bacterial clones was prepared (NucleoSpin Plasmid Kit; Machery & Nagel, Düren, Germany) and sequenced (MWG, Ebersberg, Germany).

2.3. Methylation specific PCR (MSP)

MSP was done in a total volume of 25 μl containing 1 μl bisulfite-modified genomic DNA as template. As control, universal methylated DNA (Intergen, NY, USA) was used. PCR was initiated by 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, annealing at the appropriate temperature for 45 s and 72 °C for 30 s. Final extension was done at 72 °C for 7 min. Primer were: survMeth sense 5′-TTCGGTATATTTCGCGTCGT-3′, antisense 5′-AACGTCGAAACACCCATACC-3′ (63 °C) and survUnmeth sense 5′-GGTGTGGTGTTGTTGGGTGT-3′, antisense 5′-CCAACAAATCCCACAATTCA-3′ (60 °C). MSP products were separated on 2% agarose gels and visualized after ethidium bromide staining.

2.4. Single strand conformation polymorphism analysis (SSCP)

The proximal *survivin* promoter (−266 to +63) was amplified using the primers survSSCP sense 5′-CGTTC-TTTGAAAGCAGTCGAG-3′ and antisense 5′-TGTAGA-GATGCGGTGGTCCT-3′ with following PCR conditions for 35 cycles: 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. SSCP was carried out as described previously [22]. In brief, PCR products were diluted with 85% formamide, 5% Ficoll, 0.01% bromophenol blue, heated at 95 °C for 5 min and promptly applied to 8% polyacrylamide gels with 2% glycerol. After electrophoresis at 22 °C and 200 mA for 2.5 h, products were visualized after SybrGold (Molecular Probes, Eugene, USA) or silver staining. When an abnormal migrating band was observed, the fragment was sequenced to characterize the polymorphism.

2.5. Plasmids

To amplify the proximal *survivin* promoter, genomic DNA from PBMCs (wildtype) and AML samples (with detected polymorphisms) were used as templates. The primers were: sense 5′-GCGGTACCGCGTTCTTTGAAAGCAGTC-3′ and antisense 5′-GGAAGCTTTGCCGCCGCCGCCACCT-CTG-3′ (restriction sites underlined). To specify the amplification, PCR products were excised from agarose gels and isolated using the QIAquick gel extraction kit (Qiagen) and sequenced. For reporter gene assays, the amplificates of the *survivin* promoter were *Kpn*I/*Hind*III digested and ligated into the pGL2-Basic luciferase vector (Promega). In parallel, the digested amplificates were also ligated into the pTAL-SEAP vector (BD Biosciences).