Inactivation of RUNX3/p46 Promotes Cutaneous T-Cell Lymphoma

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The key role of RUNX3 in physiological T-cell differentiation has been extensively documented. However, information on its relevance for the development of human T-cell lymphomas or leukemias is scarce. Here, we show that alterations of *RUNX3* by either heterozygous deletion or methylation of its distal promoter can be observed in the tumor cells of 15 of 21 (71%) patients suffering from Sézary syndrome, an aggressive variant of cutaneous T-cell lymphoma. As a consequence, mRNA levels of RUNX3/p46, the isoform controlled by the distal promoter, are significantly lower in Sézary syndrome tumor cells. Re-expression of RUNX3/p46 reduces cell viability and promotes apoptosis in a RUNX3/p46^{low} cell line of cutaneous T-cell lymphoma. Based on this, we present evidence that RUNX3 can act as a tumor suppressor in a human T-cell malignancy and suggest that this effect is predominantly mediated through transcripts from its distal promoter, in particular RUNX3/p46.

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INTRODUCTION

RUNX3 belongs to a family of highly conserved transcription factors and is one of the key gene expression regulators in several development processes including hematopoiesis (Bangsow et al., 2001). *RUNX3* expression is regulated by two different promoters, a distal and a proximal promoter, leading to at least two different RUNX3 proteins with DNA binding capacity (Puig-Kroger et al., 2010) (Figure 1). The RUNX3 isoform, which is derived from the distal promoter (RUNX3/p46), has 19 unique amino acids at its N-terminal end that are not present in the protein derived from the proximal promoter (RUNX3/p44). The sequence specific to the distal RUNX3 promoter transcript is highly conserved in human, mouse, and rat and comprises an independent transcriptional activation domain (Chung et al., 2007). RUNX3 plays a pivotal role in T-cell development, because it is required for the appropriate expression of CD4 or CD8 and also for T helper (Th) 1/Th2 differentiation (Djuretic et al., 2007; Naoe et al., 2007; Schulz et al., 2009; Taniuchi et al., 2002; Wang et al., 2012; Yarmus et al., 2006). Moreover, recent reports could link RUNX3 to the development of several T-cell–mediated inflammatory diseases (Apel et al., 2013; Evans et al., 2011; Jeffries et al., 2011; Lian et al., 2013; Tsoi et al., 2012).

In cancer, RUNX3 is frequently inactivated in solid tumors and its loss has an impact on apoptosis resistance and cell proliferation (Chi et al., 2005; Ito et al., 2008; Li et al., 2002; Whittle et al., 2015; Yano et al., 2006), senescence (Kilbey et al., 2008), and DNA repair (Chen et al., 2008; Tanaka et al., 2007), as well as cell adhesion or formation of metastasis and angiogenesis (Chen et al., 2013; Grueter et al., 2005; Peng et al., 2006; Sakakura et al., 2005). Moreover, it is postulated to function as a tumor suppressor whose inactivation plays a major role in, for example, gastric cancer (Li et al., 2002) and, as recently shown, in pancreatic ductal adenocarcinoma (Whittle et al., 2015). However, despite its postulated function as a tumor suppressor in many solid tumors and its pivotal role for T-cell development and differentiation, not much is known about its relevance for human hematologic malignancies.

Cutaneous T-cell lymphomas (CTCLs) are most often characterized by the proliferation of malignant CD4$^+$ T-helper memory cells displaying a Th2 phenotype in advanced stages (Willemze et al., 2005). The transcription factor RUNX3 could be of interest regarding CTCL biology for several reasons. First, RUNX3 most probably exerts its tumor suppressor function by regulating transforming growth factor-β signaling (Chuang and Ito, 2010; Ito and Miyazono, 2003). Defective or deregulated transforming growth factor-β signaling has already been shown for CTCL (Capocasale et al., 1995; Kadin et al., 2001; Lee et al., 2012; van Doorn et al., 2004). Second, RUNX3, and in particular RUNX3/p46, is crucial for the differentiation of naive

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Abbreviations: CTCL, cutaneous T-cell lymphoma; PBMC, peripheral blood mononuclear cell; SNP, single-nucleotide polymorphism; SS, Sézary syndrome; Th, T helper

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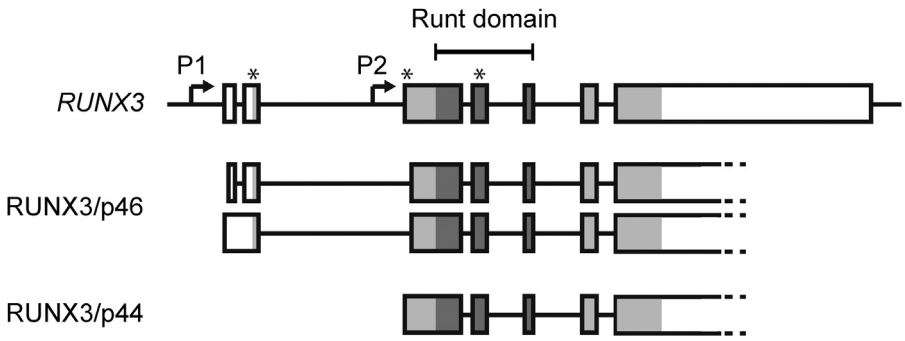

Figure 1. Structure of the human *RUNX3* locus and the transcripts coding for RUNX3/p47 and RUNX3/p44. The boxes represent the different exons. Translated regions are marked in light grey. Dark grey areas indicate the localization of the DNA-binding Runt domain. Dotted lines indicate that the untranslated part of the last exon is not entirely displayed. *Initiation codons. P1, distal promoter; P2, proximal promoter.

$CD4^+$ T cells into Th1 cells but not into Th2 (Djuretic et al., 2007; Kohu et al., 2009; Naoe et al., 2007; Schulz et al., 2009; Wang et al., 2012). Therefore, loss of function of RUNX3/p46 could lead to a predominance of Th2 cytokines, which is the case in tumor cells of CTCL (Dummer et al., 2001; Guenova et al., 2013; Lee et al., 1999; Vowels et al., 1992).

Here, we present an integrative analysis of genomic aberrations, promoter methylation, and gene expression of *RUNX3* in primary tumor cells of patients with Sézary syndrome (SS). Our results link RUNX3—in particular the long isoform of RUNX3 (RUNX3/p46)—to a human T-cell malignancy. Moreover, we show that ectopic expression of RUNX3/p46 in a CTCL cell line with very low levels of endogenous RUNX3/p46 diminishes cell viability by inducing apoptosis.

RESULTS

Genomic loss of *RUNX3* is a frequent event in SS

We recently analyzed enriched primary tumor cells from 20 SS patients by array comparative genomic hybridization (aCGH) (Steininger et al., 2011). We supplemented this data set with the analysis of three additional primary tumor cell samples and re-evaluated the combined data set with a focus on the chromosomal region 1p36. In doing so, we identified a region of frequent heterozygous genomic loss ranging from chromosome 1:25,065,561−25,241,048 (hg18). This interval was found deleted in 9 of 23 SS patient samples (39%) and the SS-derived cell line SeAx and encompasses only a single gene, which codes for the transcription factor RUNX3 (Figure 2a, Table 1). Fluorescence in situ hybridization on peripheral blood mononuclear cells (PBMCs) from five of these nine patients, the two SS cell lines SeAx and Hut-78, and two other CTCL cell lines, MyLa (mycosis fungoides) and HH (aggressive CTCL not otherwise specified), confirmed our aCGH results and indicated that the deletions are of somatic origin in all cases tested (Figure 2b, Table 1).

Point mutations within *RUNX3* are absent in SS

Next, we sequenced the coding region of *RUNX3,* including exon/intron borders, in 25 tumor cell samples and the two SS-derived cell lines, SeAx and Hut-78, but we did not find any obvious deleterious mutations. However, in 9 of 25 (36%) tumor cell samples and both SS cell lines we detected a nonsynonymous single-nucleotide variant that corresponds to an inherited single-nucleotide polymorphism (SNP) (rs6672420), which leads to an amino acid exchange at position 18 of RUNX3/p46 from isoleucine to asparagine (Table 1). A synonymous SNP (rs143410594) was found in one patient. All variants were found in patient samples that did not show heterozygous deletion of *RUNX*3. Sequencing of *RUNX3* in isolated monocytes from six of those nine patients showed that all polymorphisms were of germ line origin (Table 1).

Methylation of the distal, but not the proximal, promoter is a specific feature of SS cells

Because the proximal *RUNX3* promoter is frequently methylated in various solid tumors, we first analyzed its methylation status by bisulfite sequencing in 19 tumor cell samples, $CD4^+$ T cells of 10 healthy volunteers, the four CTCL cell lines, and five T-cell acute lymphoblastic leukemia cell lines (Jurkat, Molt-4, KE-37, CCRF, and K45). We did not find promoter methylation in any of the samples (data not shown). We then proceeded to analyze the distal *RUNX3* promoter, including five additional SS samples. Although all samples from healthy volunteers and all T-cell acute lymphoblastic leukemia cell lines did not show any promoter methylation, in 6 of 24 SS samples, the analyzed CpG sites were mainly or completely methylated (>70% methylation of analyzed CpG sites), and 5 of 24 samples showed a mixed methylation pattern, resulting in an overall methylation frequency of 46% in the analyzed patient samples (Table 1 and Figure 2c). Of the four tested CTCL cell lines, we detected methylation of the distal promoter in MyLa cells, in which the analyzed CpG sites were mainly methylated.

Cytoplasmic retention of RUNX3 protein is not observed in CTCL

To test if cytoplasmic retention of the protein might be another mechanism of RUNX3 inactivation in SS or other entities of CTCL, we stained RUNX3 in our set of CTCL cell lines and Jurkat cells by immunofluorescence staining. Although all of the five cell lines stained positive for RUNX3 with different intensities, none of them showed an obvious cytoplasmic retention of the protein that could be verified by Western blot analysis of nuclear and cytoplasmic protein extracts (Figure 2d and e).

RUNX3/p46 expression is reduced in SS tumor cells

We next tried to determine whether our genomic findings are reflected by a reduced *RUNX3* expression in SS tumor cells. Immunohistochemical staining of RUNX3 in 10 skin biopsy samples from SS patients—four with and six without genomic loss of *RUNX3*—did not show reduced RUNX3 protein levels