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ScienceDirect
Leukemia Research 30 (2006) 1547–1553
Leukemia Research
www.elsevier.com/locate/leukres

Aberrant DNA demethylation in promoter region and aberrant expression of mRNA of PAX4 gene in hematologic malignancies

Yinghua Li [a], Hirokazu Nagai [a,*], Toshihito Ohno [b], Haruhiko Ohashi [a], Toyoaki Murohara [c], Hidehiko Saito [a], Tomohiro Kinoshita [b]

[a] *Clinical Research Center for Blood Diseases, National Hospital Organization Nagoya Medical Center, 4-1-1, Sannomaru, Naka-ku, Nagoya, Japan*
[b] *Department of Hematology, Nagoya University, Graduate School of Medicine, Nagoya, Japan*
[c] *Department of Cardiology, Nagoya University, Graduate School of Medicine, Nagoya, Japan*

Received 4 February 2006; received in revised form 31 March 2006; accepted 4 April 2006
Available online 15 May 2006

Abstract

The PAX4 gene, a member of the paired box (PAX) gene family, is thought to be involved in regulating the fate of β-cells in the mammalian pancreas. We observed the aberrant expression of PAX4 mRNA in 10 of 15 hematologic cell lines analyzed by RT-PCR. The restoration of PAX4 gene expression after treatment with the demethylating agent 5-aza-2′-deoxycitidine, as well as bisulfite sequencing analysis, indicated that gene overexpression was caused by DNA demethylation at the promoter region. Such DNA demethylation also was observed in primary lymphoma (20 out of 45 patients) on combined bisulfite restriction assay (COBRA). Forced expression of the PAX4 gene in the HEK293 and SHSY/610 cell lines conferred positive effects on cell growth. This profile of PAX4 thus corresponds to that of a candidate oncogene in hematologic malignancies.

Keywords: PAX4; Homeobox gene; Lymphoma; DNA methylation; Bisulfite modification; Oncogene

1. Introduction

Paired box (PAX) genes encode a family of transcription factors that contain a paired box domain and have essential roles in the development of multicellular organisms [1,2]. The paired box domain, which contains a DNA-binding motif, was originally identified in the *Drosophila* segmentation genes *paired* [1], *gooseberry distal* (*gsb-d*) and *gooseberry proximal* (*gsb-p*), and has been highly conserved during evolution. To date, nine members of the PAX gene family have been identified in the human and murine genomes (PAX1–9) [3]. Three PAX genes have been shown to be associated with inherited human diseases (PAX2, renal-colobomas syndrome [4]; PAX3, Waardenburg syndrome [5,6]; PAX6, aniridia [7]), indicating the essential roles of PAX gene family in developing organs.

Similarly to other homeobox genes, some PAX genes have been investigated as oncogenes [8]. In fact, PAX3 [9,10] and PAX5 [11,12] are targets of chromosomal translocations in human malignancies. PAX5 is located on chromosome 9p13, and t(9;14)(p13;q32) translocation may cause the juxtaposition of this gene to the IgH locus in lymphoplasmacytoid lymphoma, resulting in deregulated expression of the PAX5 gene. Overexpression of some PAX genes has also been observed in human neoplasms, such as medulloblastoma, ovarian cancer and thyroid cancer [13–15]. These findings suggest that abnormal expression of PAX genes might contribute to tumor development. The oncogenic potential of PAX1, 2, 3, 6 and 8 has been demonstrated by transforming assay, and was suggested to be dependent on the DNA-binding function of the paired box domain [16].

The PAX4 gene is classified into the same group (class IV) as PAX6 because its structure contains a paired domain and a homeo domain, lacking an octapeptide [17]. PAX4 is required for differentiation of insulin-producing β-cells in

* Corresponding author. Tel.: +81 52 951 1111; fax: +81 52 951 9075.
E-mail address: nagaih@nnh.hosp.go.jp (H. Nagai).

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doi:10.1016/j.leukres.2006.04.001

the pancreas [18], but its function in the developed individual remains unclear. PAX4 is located on chromosome 7q32 [19]. This site has been reported to be a frequent target of chromosomal alterations in human tumors, including hematologic malignancies. Additions, deletions and translocation breakpoints at chromosome 7q32 were observed frequently especially in lymphoid malignancies [20–22]. The potential oncogenic function as well as the chromosomal location of PAX4 has prompted us to screen this gene as a candidate oncogene.

2. Materials and methods

2.1. Samples and DNA/RNA preparation

DNA was isolated from the biopsied lymph nodes of 45 patients with diffuse large B cell lymphoma (DLBCL) and 4 patients with reactive lymphadenitis. In addition, we isolated DNA from the peripheral blood mononuclear cells (PBMNCs) of four healthy volunteers as described elsewhere [23]. All patients gave informed consent to participate in the study. We also examined 15 hematological cell lines (NAMALWA, RAJI, RAMOS, ARA10, HS-SULTAN, KMS-12-PE, MOLT4, BALL1, JURKAT, NALL-1, NALM-17, KOPT-K1, HL60, CMK86, KU812F). RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. RT-PCR

First-strand cDNA was synthesized from 1 μg of total RNA using random hexamers as primers and MMLV-H-reverse transcriptase (Gibco-BRL, Rockville, MD). RT-PCR proceeded as described previously using first-strand cDNA as a template [23]. Briefly, 40 PCR cycles proceeded at an annealing temperature of 56 °C using the primers PAX4-F at exon 6 (5′-ACAGAAGAGCCAAATGGCGT-3′) and PAX4-R at exon 8 (5′-TGTCACTCAGACACCTTTCT-3′) to generate 222-base pair (bp) products. PCR products were separated on 2% agarose gels and were visualized by ethidium bromide staining. The control was GAPDH cDNA amplified by RT-PCR (25 cycles) in separate tubes from PAX4 gene amplification using the primers GAPDH1 (5′-CCATGGAGAAGGCTGGGG-3′) and GAPDH2 (5′-CAAAGTTGTCATGGATGACC-3′) to generate 225 bp products. PAX4-F, PAX4-R and GAPDH primers were designed not to amplify genome sequences.

2.3. Culture with 5-aza-2′-deoxycitidine

RAJI and HL60 cells (2×10^5/ml) were incubated with 5-aza-2′-deoxycitidine (0, 5, or 10 μM) for 24 h on day 2 after seeding. Cells were washed and incubated for a further 72 h and were harvested on day 5 for analysis of PAX4 gene expression and DNA methylation.

2.4. Bisulfite sequencing

We modified 500 ng of genomic DNA extract from clinical samples and cell lines with sodium bisulfite, as described elsewhere [24], using a DNA modification kit (CpGenome DNA Modification Kit; Oncor, Gaithersburg, MD) according to the manufacturer's instructions. Modified DNA (25 ng) was amplified by PCR to obtain the promoter sequence of the PAX4 gene. The PCR consisted of 35 cycles at an annealing temperature of 56 °C, using the primers PAX4-MF1 (5′-GTATGGTGGTTTGTGGGATT-3′) and PAX4-MR1 (5′-AAACTCTAATCCTACACACT-3′) (Fig. 1). Under these conditions, we were able to assess the DNA methylation status of PAX4 at 1347–1103 bp upstream of the 1st codon. PCR products were separated on 2% low melting agarose gels, excised and digested with β-agarase (New England Biolabs, Beverly, MA). Digestion products were subcloned into the PGEM-Teasy vector (Promega, Madison, WI), and at least 10 clones of each product were subjected to cycle-sequencing (PE Applied Biosystems, Warrington, UK) and analyzed using an ABI 310 (Applied Biosystems, Foster City, CA).

2.5. Combined bisulfite restriction analysis (COBRA)

In order to screen larger numbers of samples for PAX4-specific demethylation, we used COBRA [25], which produces restriction fragments from bisulfite-treated DNA only if demethylation is present. DNA modified with sodium bisulfite was selectively amplified by PCR using the primers PAX4-MF1 and PAX4-MR1 under the conditions described above, followed by 2nd round PCR with PAX4-MF2 (5′-TGTTAGTTGAATGAATGAGTGTT-3′) and PAX4-MR2 (5′-CATCTCTTAACATCTCTAACCTA-3′) (Fig. 1). Conditions for 2nd round PCR were the same as for 1st round PCR. Under these PCR conditions, we obtained DNA fragments of 124 bp, which were cleaved into fragments of 88 and 36 bp by digestion with *Ssp*I when CpG (shown as target CpG for COBRA in Fig. 1) was demethylated.

2.6. Cell culture and generation of stable transformants

HEK293 and SHSY/610 cell lines were grown in minimum essential medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum. Cell lines were plated at 2×10^5 cells/ml in 6-well plates and transfected with full length PAX4 subcloned into the pCDNA3.1/His vector (Invitrogen, Carlsbad, CA) (kind gift from Dr. Miyamoto, Shinshu University School of Medicine), or the pCDNA3.1/His vector alone (mock) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. At 48 h after transfection, selection was initiated using G418 (Sigma–Aldrich) at a concentration of 300 μg/ml. PAX4 expression was evaluated by Western blotting against histidine tags.